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Oxidative stress and chronic inflammation in osteoarthritis: can Nrf2 counteract these partners in crime?

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Short title: The regulatory role of Nrf2 in osteoarthritis

Keywords: Osteoarthritis; Nrf2; ROS; inflammation; phytochemicals

Abstract

Osteoarthritis (OA) is an age-related joint degenerative disease associated with pain, joint deformity and disability. The disease starts with cartilage damage but then progressively involves subchondral bone causing an imbalance between osteoclast-driven bone resorption and osteoblasts-driven remodeling. Herein we summarize the data for the role of oxidative stress and inflammation in OA pathology and discuss how these two processes are integrated during OA progression, as well as, their contribution to abnormalities in cartilage/bone metabolism and integrity. At the cellular level, oxidative stress and inflammation are counteracted by transcription factor nuclear factor-erythroid p45-related factor 2 (Nrf2), and we describe the regulation of Nrf2, highlighting its role in OA pathology. The beneficial effect of some phytonutrients, including the therapeutic potential of Nrf2 activation, in OA is also discussed.

Introduction

Osteoarthritis (OA) is one of the most common degenerative arthropathy in the elderly population. OA is a time- or age-dependent progressive destruction of joints related to articular cartilage damage, synovial lining thickening, subchondral bone alterations, osteophytes formation at the joint margin, and is supported by elevated inflammatory and catabolic responses, which finally result in a loss of joint architecture and deformity.[1-4] The disease affects mostly individuals over the age of 50 (primary disease), but it can also develop after joint insult and trauma in younger individuals (post-traumatic OA). Several lines of evidences suggest that aging has an impact on the pathogenesis of primary disease. Some OA chondrocytes acquire aging phenotype with increased genomic instability, telomere shortening, epigenetic alterations including decreased genomic global methylation and histone modifications, dysregulated metabolism, mitochondrial dysfunction and altered intercellular networks and cascades.[5-7] The disease progresses and the age-dependent alterations in cell signaling and metabolism occur in chondrocytes but also in bone cells (osteoclasts and osteoblasts), causing an imbalance between bone resorption and remodeling and deeper abnormalities in subchondral bone and joint structure. Aging causes OA development, however it is not a sole risk factor. The disease is multi-factorial with both physiological and mechanical processes involved, such as obesity, genetic predisposition, hereditary, metabolic or endocrine alternations, trauma, joint overload and mechanical stress, as well as, long-time exposure to a low-grade chronic inflammation concomitant with a failure in oxidant-antioxidant balance.[5-8] Therefore, some aspects of OA pathology are still a subject of an extensive research.[4,9]

Inflammation and oxidative stress have been increasingly recognized as being closely integrated in OA pathology. Various inflammatory mediators such as cytokines [tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, 8, 6, 15, 17, 18, 21, 33],[10] chemokines, prostaglandins (PGEs)[11] and growth factors [transforming growth factor-α (TGF-α)], fibroblast growth factor (FGF) are increased in the joint tissue of OA patients in comparison to healthy individuals.[9] Local inflammatory response along with aging and/or mechanical load can contribute to increased oxidative stress with accumulation of reactive oxygen species[12] (ROS), superoxide anion, hydrogen peroxide (H_2O_2), nitric oxide (NO) and peroxynitrite and concomitant failure in the expression of antioxidant enzymes and ROS scavenging systems.[13] At cellular level, oxidative stress causes mitochondrial DNA (mtDNA) and nuclear DNA damage, lipid peroxidation, alterations in cell signaling, transcription and epigenetic changes in gene expression. At the level of the joint as a whole organ, oxidative stress causes

abnormalities in the cartilage and bone metabolism, exacerbating not only degradation but also overall reparative potential of chondrocytes, osteoblasts and their precursors.[13,14] Thus the regulation of redox homeostasis at cellular level can decrease the severity of OA and limit the disease progression.

The Cap'n'Collar basic leucine zipper transcription factor nuclear factor-erythroid 2 p45-related factor 2 (Nrf2, also called Nfe2l2) is the master regulator of the cellular redox homeostasis. Nrf2 regulates the expression of a large network of cytoprotective genes, including enzymes involved in the biosynthesis, utilization and regeneration of reduced glutathione (GSH), thioredoxin and nicotinamide adenine dinucleotide phosphate (NADPH), detoxification enzymes such as glutathione S-transferases (GST), aldo-keto reductases (AKR), and NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HO-1), as well as proteins involved in the recognition and clearance of damaged proteins and organelles, such as proteasomal subunits and autophagy-related proteins.[15-16] Collectively, the products of the Nrf2-target genes function to lower the intracellular levels of ROS, reactive nitrogen species (RNS) and electrophiles, thus protecting the cell from the harmful effects these agents. In addition, Nrf2 is involved in the regulation of expression of a number of metabolic genes, most notably those involved in the pentose phosphate pathway, the biosynthesis of purine nucleotides and serine, and enhance fatty acid metabolism and mitochondrial function.[17-21] Overall, the Nrf2 regulatory network provides an interface between redox and intermediary metabolism.

Recently, several studies have revealed the essential role of Nrf2 in preventing the patho-physiological events in joint diseases.[22] It has been shown that the appropriate expression and fine balance in Nrf2 activity is absolutely necessary for normal chondrogenesis and proper regulation of cartilage metabolism.[12,23] There is growing evidence for the involvement of Nrf2 in bone resorption and remodeling indicating a multi-faceted effect in sustaining overall bone integrity. Herein, we attempt to summarize the recent findings for the essential role of Nrf2 in OA. We also discuss the underlying mechanisms of regulation and biochemical crosstalk between Nrf2, ROS signaling and inflammation during OA pathogenesis. The beneficial role of phytonutrients to attenuate cartilage/bone degradation, inflammation and oxidative stress in OA is highlighted.

Nrf2 function and regulation

Nrf2 is expressed in most cell/tissue types and comprises seven functional domains, arranged as follows from its N- to C- terminus: Neh2, Neh4, Neh5, Neh7, Neh6, Neh1, Neh3 **(Figure**

1). The Neh4 and Neh5 domains form the transactivation domain of the transcription factor that recruits CREB-binding protein (CBP). The retinoid X receptor α (RXRα) represses Nrf2 by binding to its Neh7 domain. Neh1 mediates binding to DNA and to the heterodimeric partner of Nrf2, transcription factor of the family of small musculoaponeurotic fibrosarcoma (Maf) proteins. Neh3 recruits the chromo-ATPase/helicase DNA-binding protein 6 (CHD6). The Neh2 and the Neh6 domains contain specific sequences (degrons) that mediate the degradation of Nrf2.[24] Nrf2 is regulated primarily at the level of protein stability. At homeostatic conditions, the levels of Nrf2 are low due to its continuous ubiquitination and proteasomal degradation. In response to stress signals, Nrf2 accumulates, translocates to the nucleus, and as a heterodimer with a small Maf, initiates transcription of its target genes.[24]

There are three known ubiquitin ligase systems, which have been implicated in the degradation of Nrf2 **(Figure 2)**. The major negative regulator of Nrf2 activity is Kelch-like ECH-associated protein 1 (Keap1). Keap1 was identified as a result of studies which followed on from the finding that deletion of the Neh2 domain of Nrf2 (to which Keap1 binds) resulted in its greatly increased activity.[25] Subsequent research has elucidated the process by which Keap1 downregulates the Nrf2 activity. Keap1 forms a homodimer via its BTB domain.[26-28] The regions of Keap1 important for interacting with Nrf2 are its Kelch (also known as double glycine repeat, DGR) domains and C-terminal regions, and each Keap1 molecule interacts with one of two proximal portions of Nrf2, the DLG and ETGE motifs **(Figure 1)**.[29] Both of these motifs lie within the Neh2 domain of Nrf2, separated by an alpha helix, and due to their different electrostatic potentials, the ETGE motif has an approximately 100-fold higher affinity for Keap1 than the DLG motif.[30] A "hinge and latch" hypothesis has been proposed whereby the low affinity site (latch) serves to position Nrf2 such that its lysine residues are optimally oriented for ubiquitination, while the high affinity site (hinge) is primarily responsible for keeping Nrf2 bound to Keap1.[31] Keap1 uses a cyclic mechanism to sequentially bind to the ETGE and the DLG motifs of Nrf2.[32] While bound to both of these sites of Keap1, Nrf2 is in a prime position for ubiquitination by Cullin-3 (Cul3)-Rbx1/Roc1 ubiquitin ligase (*i.e.*, CRL^{Keap1}), which is recruited to the complex via interactions with the BTB domains of the Keap1 dimer.[28] Ubiquitinated Nrf2 is extracted from the Keap1-Cul3 E3 complex by the ATP-dependent segregase p97,[33] and degraded by the 26S proteasome - this regulation maintains the half-life of Nrf2 at 10-20 minutes, and ensures low basal Nrf2 activity.[29]

Besides acting as the primary inhibitor of Nrf2 activity, Keap1 serves as the intracellular sensor for electrophiles and oxidants,[27] which bring about stabilisation and

activation of Nrf2. This is mediated through specific reactive cysteine residues of Keap1, some of which have lower *pKa* values than most cysteine residues due to their surrounding basic amino acids, meaning they exist as thiolate anions (S^-, as opposed to SH) making them more reactive with ROS, RNS and electrophiles. Which cysteine residues are modified depends on the type of activator,[34] with C151, C273 and C288 being most commonly modified.[35-37] Small molecule electrophiles and oxidants chemically modify Keap1 cysteine residues, which is hypothesized to alter the structure of the complex, preventing ubiquitination and subsequent degradation of the transcription factor. This results in Keap1 becoming saturated with Nrf2 and allowing newly synthesised Nrf2 to accumulate in the cytoplasm, translocate to the nucleus and induce expression of its target genes.[32] Alternatively, some toxic metals (As^{3+}, Cd^{2+}, and Cr^{6+}) have been found to stabilise Nrf2 by dissociating it from Keap1.[38] Other small molecule Nrf2 activators directly disrupt the Keap1 : Nrf2 protein : protein interaction.[39,40]

Upon translocation to the nucleus, Nrf2 forms heterodimers with a small Maf protein, and this dimerization is necessary for efficient DNA binding.[41] Nrf2-Maf heterodimers bind their target gene promoters at antioxidant response elements (ARE), which comprise the core sequence 5′-TGACnnnGC-3', in which the GC dinucleotide is a critical part.[42] Both of these transcription factors contain a basic leucine zipper (bZIP) motif found within their DNA-binding domains, with the basic region contributing to DNA binding and the leucine zipper facilitating dimerization.[38] As mentioned above, the Neh4, Neh5 and Neh3 domains of Nrf2 **(Figure 1)** are responsible for mediating transactivation of target genes by recruiting a number of coactivators, whereas the small Maf has no transactivation domain, and does not contribute to this process.[43] Coactivators recruited include CREB-binding protein (CBP), coactivator-associated arginine methyltransferase 1 (CARM1) and protein arginine methyl-transferase 1 (PRMT1), which appear to synergise with another recruited coactivator, receptor associated co-activator 3 (RAC3) to facilitate Nrf2-mediated gene expression.[44] Other studies have unveiled more bZIP binding partners, with which Nrf2 dimerizes, including activating transcription factor 4 (ATF4), Fos and Jun.[45] While these heterodimers have been shown to bind to the ARE, it has not yet been made clear the extent to which they participate in regulating gene expression. Additionally, Nrf2 transactivation is subjected to negative regulation by retinoic X receptor α (RXRα), which binds Nrf2 and sits at the ARE to prevent binding of Nrf2-Maf heterodimers.[46] A recent study has shown that Nrf2 binds to MED16,[47] a tail subunit of Mediator, a highly conserved large conformationally flexible macromolecular complex, which is required for RNA polymerase II-driven transcription of all protein-coding

genes.[48,49] Pull-down assays have indicated that the Neh4/Neh5 and the Neh1 domains of Nrf2 are involved in this interaction.[47]

In addition to Keap1, Nrf2 is targeted for degradation following glycogen synthase kinase 3 β (GSK3β)-mediated phosphorylation **(Figure 2)**.[50,51] This kinase phosphorylates serine residues in the Neh6 domain of Nrf2, leading to the recruitment, through DSGIS and DSAPGS motifs **(Figure 1)**, of the dimeric β-transducin repeat-containing protein (β-TrCP), a substrate adaptor for the Skp1-Cul1-Rbx1/Roc1 E3 ligase complex (*i.e.*, $SCF^{\beta\text{-}TrCP}$), hence resulting in the degradation of the transcription factor. Activation of certain kinase cascades, including phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways, leads to inhibition of GSK3β, and certain Nrf2 activators such as the lignan nordihydroguaiaretic acid (NDGA), have been shown to activate Nrf2 via induction of these pathways and GSK3β inhibition.[52] A third ubiquitin ligase system, the endoplasmic reticulum (ER)-bound E3 ligase Hrd1 (also known as synoviolin), has been implicated in the degradation of Nrf2 during ER stress **(Figure 2)**.[53] Acetylation also plays a role in Nrf2 regulation – the histone acetyl transferases (HAT) p300 and CBP modify lysine residues of Nrf2 within its Neh1 domain to promote its DNA-binding capacity at certain target genes.[54]

The activity of Nrf2 can be affected by transcriptional and epigenetic mechanisms. Thus, Nrf2 transcription is upregulated by the arylhydrocarbon receptor (AhR) and BRCA1,[55,56,57] whereas it is downregulated in the livers of peroxisome proliferator-activated receptor α (PPARα)-null mice following a 24-h period of fasting.[58] In addition, Nrf2 is upregulated in tumor cells with mutant $K\text{-}Ras^{G12D}$ or $B\text{-}Raf^{V619E}$, and it has been proposed that this upregulation is mediated by transcription factors Jun and Myc.[59] Nrf2 can also activate its own transcription due to the presence of an ARE-like sequence (5′-CTGACTCCGCC-3′) in its promoter.[60] The promoter of the murine *Nrf2* gene contains five CpG sequences, which have been shown to be hypermethylated in a mouse model of prostate cancer, resulting in a transcriptional silencing of Nrf2 and lower target gene expression.[61]

Accumulating experimental evidences suggest that micro-RNAs (miRs) also play a role in the regulation of Nrf2. In human MCF-7 breast cancer cells, ectopic expression of miR-28 leads to a decrease in the mRNA and protein levels of Nrf2.[62] Overexpression of miR-93 has also been shown to reduce the levels of Nrf2 (mRNA and protein) as well as its downstream target gene NQO1 in human MCF-10A and T47D breast cancer cells.[63] Conversely, the downregulation of miR-93 increased the expression of Nrf2 and its target gene heme oxygenase 1, and had a protective effect in a mouse model of stroke.[64] In human SH-SY5Y neuroblastoma cells, ectopic expression of miR-27a, miR-142-5p, miR-144 and

miR-153 results in a decrease in the mRNA and protein levels of Nrf2 and the expression of NQO1.[65] Downregulation of miR-27a increased Nrf2 expression and improved cardiac function in a mouse model of LPS-mediated sepsis.[66] Interestingly, in homozygous sickle cell disease (HbSS) reticulocytes, increased levels of miR-144 are inversely associated with the levels of Nrf2, and correlate with decreased glutathione regeneration and attenuated antioxidant capacity in HbSS erythrocytes.[67] A recent study has shown that miR-144 reverses chemoresistance in hepatocellular carcinoma cell lines by targeting the 3'-untranslated region (3'-UTR) of Nrf2 and promoting its mRNA degradation.[68] By a similar mechanism, miR-153 downregulates Nrf2, whereas tanshinone IIA, a diterpene quinone from *Salva miltiorrhiza*, restores this downregulation, leading to protection of SH-SY5Y cells against 6-hydroxydopamine-induced toxicity.[69]

Pathogenesis of osteoarthritis

Osteoarthritis is a worldwide problem accounting for 1.1% of global disability-adjusted life years[10] and the fourth leading cause of disability,[22] accompanied by pain, and depression, with social impact on the patients' families.[4,5,9] Currently, available therapies are limited in efficacy because of the complex pathology and attended side effects.[70] Most therapies only aim to reduce and control OA symptoms and pain, but they fail to provide a cure and thus total joint replacement is often required in the most severe cases of OA.[22,10,70]

At the early stage of disease the changes in chondrocyte function and survival lead to progressive cartilage disintegration and deterioration. In some patients this process can be accompanied and perpetuated by secondary inflammation and synovitis.[71] Cartilage degeneration accelerates catabolic processes and bone removal by osteoclasts (bone resorption) resulting in bone cysts and sclerotic bone formation. Cartilage and bone loss trigger compensatory repair by osteoblasts with excessive anabolic processes of neocartilage formation at the joint margins and joint edges (osteophytes), periarticular fibrosis and calcified bone formation in advanced disease. The repair at specific site of the joints is a consequence also of generalized alteration in the intrinsic capacity of subchonrial bone to repair the cartilage and in the cross-talk between immune and bone cells.[8]

Despite that OA can be diagnosed quickly by imaging techniques, the disease progresses slowly within years before the first symptoms and a risk score for the individual's susceptibility to OA can be predicted based on analysis of genetics, age and environmental factors in combination with molecular and imaging biomarkers.[8] Such prediction can be

based on the assumption that the primary disease develops as a *continuum*. Mutations and errors in gene expression of matrix molecules or factors regulating matrix component synthesis can render chondrocytes hypertrophic and dysfunctional and can cause chondrodysplasia at a relatively early age.[8] Chondrocytes can acquire this altered phenotype progressively especially when they are situated near weight-bearing regions. Over-time (at middle-age; with aging) OA susceptibility can increase because of supplementary mechanical and structural disorders in the joints associated with reduced physical activity and muscle strength, altered bone metabolism, infections, joint injury, inflammation and unhealthy diet.[8] Finally, age-related changes in growth factor signaling required for morphogenesis and repair contribute to OA onset in the elderly population.[8] At middle- and late-onset, different cell types can be involved in the pathology – chondrocytes, synovial fibroblasts, bone and immune cells, and all of them may potentially show aberrant gene expression and a failure in oxidant-antioxidant balance. With respect to secondary disease, increased susceptibility to develop OA might be related to altered epigenetic modifications of cartilage after injury. Indeed, more than 1000 differentially methylated regions (DMRs) have been detected in human knee articular cartilage upon damage.[72] Additional impact in the secondary disease is chronic inflammation often related to dysfunction in immune mechanisms and/or immune cell homeostasis.[73]

Role of inflammation and oxidative stress in osteoarthritis

Inflammation and interaction between immune and bone cells in OA

One unresolved link in primary OA is the contribution of inflammation in disease initiation and progression. Several studies have shown a significant association between OA severity and the presence of inflammatory synovitis).[71,74] Besides OA synovial fibroblasts, OA chondrocytes show altered expression of inflammatory genes, of genes encoding transcription factors or regulatory genes and of genes, associated with the loss of correct cell maturation.[75,76] Indeed, overexpression and overproduction of pro-inflammatory cytokines have been clearly shown to be part of OA pathology.[9,10,11] In chondrocytes, cytokines activate PI3K/Akt and NF-kB signalling pathways, up-regulate matrix metalloproteinases (MMPs)/aggrecanases expression and increase DNA hydroxymethylation via attenuation of ten-eleven translocation 1-3 (TET1-3) enzymes.[77] OA chondrocytes can also produce chemokines, such as IL-8, that drive neutrophil accumulation.[78,79]

Indeed, neutrophils can impact seriously OA pathology. It has been found that blood neutrophils from OA patients with active disease respond to in vitro stimulation with elicited pro-inflammatory cytokine production and show elevated expression of bone-damage accelerating receptor activator of nuclear factor-κB (RANKL).[80] Mature neutrophils expressing RANKL are found in synovial fluid of mice with OA[81] and disease improvement is associated with inhibition of RANKL expression on neutrophils.[82] Neutrophils actually endow a destructive phenotype in blood that is further extended and sustained in arthritic synovium.[83] In turn, they can drive osteoclast differentiation and maturation from precursor cells directly via RANKL-RANK pathway or they can manipulate chondrocyte and osteoblast functions indirectly via ROS, cytokines and growth factors secretion. Neutrophils can cooperate with NK cells to build the local immune response and can induce general changes in NK homing and activation via secreted chemokines[73] that in turn can fail to support normal cartilage and bone repair. It is still unclear if some of the abnormalities in advanced OA are related to age-dependent dysfunction in immune cells and ROS signaling as well as with a shift in the expression of myeloid lineage genes and changes in the composition and quality of the tissue-restricted pools of immune cells.[84]

Role of oxidative stress in OA pathology

During the past decades, tremendous progress has been made in understanding the role of oxidative stress in health and diseases. ROS including H_2O_2, $O_2\bullet^-$, and •OH are byproducts of normal cellular metabolism. ROS are generated during electron transport chain reactions delivering electrons to molecular oxygen in the mitochondria. The process is restricted by the redox enzyme $p66^{Shc}$, which is translocated in the mitochondria in response to exogenous signals delivered by growth factor deprivation, oxidative stress or UV irradiation.[85,86] Generated H_2O_2 can diffuse across the outer mitochondrial membrane to the cytosol where it can modulate the activity of "ROS sensors" such as the thiol functional group of the amino acid cysteine in proteins. Subsequently, the oxidative cysteine modifications can modulate cytosolic protein functions, akin to phosphorylation and can increase mitochondrial permeability and biogenesis.[86] H_2O_2 also downregulates the activity of the forkhead transcription factor FoxO3, implicated in the expression of mitochondrial antioxidant enzymes, that in turn increase both hydrogen peroxide scavenging and oxidative stress resistance.[87]

ROS generation in mitochondria can often change during aging resulting in persistent endogenous ROS production at small amounts contributing to aging-related mitochondrial

dysfunction, swelling and apoptosis together with alterations in chondrocyte senescence and apoptosis. This pathway is considered to play the minor role in the total ROS accumulation but it has the key implication in aging-dependent alterations in OA.[14,22]

The significant amounts of ROS are generated via non-mitochondrial pathway, *i.e.* NADPH oxidase (NOX) or dual oxidase (DUOX) assembles found at the discrete regions at the plasma or endosomal membranes. In phagocytic cells, the active oxidase contains the catalytic subunit (gp91phox), the regulatory subunits (p22phox, p47phox, p40phox, p67phox) and the small signaling G protein kinase. Later it was discovered that non-phagocytic cells from various tissues can express one of the seven homologues of the gp91phox: NOX 1-5 and DUOX 1-2, all containing conserved domain structure of six transmembrane α-helices and an electron transfer center with two haems forming the electron transport channel through the membrane.[88] The C-terminal domain of the multi-component oxidase binds to the electron-donating pyridine nucleotide NADPH and to the various cofactors such as flavin adenine dinucleotide (FAD) or Ca^{2+} in order to generate superoxide. In a subsequent reaction, two molecules of superoxide can generate H_2O_2. Similarly to the mitochondrial pathway, H_2O_2 can induce reversible modifications of cysteine containing proteins like tyrosine phosphatases adjacent to receptors for growth factors, cytokines and chemokines or like phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (PTEN) responsible for the hydrolysis of the bioactive lipid phosphatidylinositol 3,4,5-trisphosphate. In such a way, the NOX enzymes can regulate the downstream signaling for cell activation, differentiation, proliferation and apoptosis in healthy cells. H_2O_2 can diffuse in the cytosol but NOX complexes are present at the lipid rafts[89] and assemble at focal adhesion regions,[90] restricting H_2O_2 to specific cellular micro-domains and preventing aberrant signaling.

Another system for ROS generation involves the constitutively expressed enzyme xanthine oxidoreductase (XOR). XOR is highly expressed in intestine and liver and also in synovium, lung, kidney and brain. The XOR activity is versatile and tuneable at multiple-levels.[91] At genetic level the expression of the enzyme can be regulated by nutritional factors, oxygen tension, steroid hormones, phorbol esters, regenerative and hyperplastic stimulus, cytokines implicated in inflammation (like IFN-γ and TNF-α)[91]. The enzyme can exist in two forms - dehydrogenase and xanthine oxidase (XO) as it undergoes irreversible partial proteolytic cleavage or reversible post-translational modifications via thiol oxidation.[92,93] During the latter convertible alteration, XOR enzyme is modified from dehydrogenase via intermediate form to reversible XO.[91] The conversion of XOR to XO has been observed in a

variety of hypoxic and ischaemic conditions in the tissues including synovium and has been related with vascular response to oxidative stress and the regulation of pro-inflammatory and pro-thrombotic activities of endothelial cells.[91] The mechanisms include the metabolic alterations and the activation of transcriptional factors, including hypoxia-inducible factor-1 (HIF-1) and protein kinases.[94] XOR can catalyze the oxidation of hypoxanthine to xanthine, can act as a NADH oxidase (the XO form) to catalyze the subsequent oxidation of purines to urate and can catalyze the reduction of nitrates and nitrites, being the rate-limiting enzyme in purine catabolism as well as being the constant source of ROS, NO and peroxynitrite.[92,93,95,96] XOR level in synovial fluid is elevated in OA patients but in a lesser extent in comparison to that in patients with rheumatoid arthritis,[97] and its amount in synovium correlates with the severity of joint injury[97-99] and inflammasome activation driving OA progression.[100] More than 50% of synovial XOR exists in oxidase form and is expressed in synovial tissue upon injury.[97,101] Increased XO activity is associated also with altered adhesion and accumulation of leukocytes in microvasculature and elevated ROS and cytokine production by infiltrating and locally activated monocytes and neutrophils.[91]

Several scavenging systems are available in the cells to detoxify ROS: the enzymes superoxide dismutases (SOD), catalase, glutathione peroxidase and glutathione reductase.[102] SOD1 is a dimeric cytosolic enzyme that binds copper and zinc (Cu/ZN-SOD), whereas SOD2 is a mitochondrial homo-tetramer that binds one manganese ion per subunit (Mn-SOD). Both enzymes convert superoxide to H_2O_2 and diatomic oxygen. Catalase is a tetrameric protein that converts H_2O_2 to H_2O and gaseous O_2. In the cytosol glutathione peroxidase/glutathione reductase system maintains the reducing environment in the cells. When ROS production escapes the antioxidant systems and mechanisms, the cells are exposed to oxidative stress and become sensitive to the activation of apoptotic pathways. ROS-mediated damage by oxidative stress can be often reversed by repair, replacement, degradation or sequestration of the damaged macromolecules, but in some cases the stress can be sustained driving mitochondrial and cell death or mutagenesis.[85,86] The biological effects of ROS in all cell types are due to hyperperoxidation, protein carbonylation, direct DNA damage, telomere shortening, epigenetic alterations of gene expression and failure in DNA repair, changes in receptor and metabolic pathways and autophagy. However, the sources of ROS may differ in various cell types and may be dependent on cell type functional and metabolic state.

Abnormal ROS signaling has been described in OA synovial tissue, fibroblasts chondrocytes, osteoblasts and osteoclasts, concomitant with spatiotemporal progression of

injury from the articular surface to the subchondral bone. ROS generation can be influenced by underlying chronic inflammation involving immune cells or by generalized alterations in cell-driven repair affecting nuclear reprogramming (**Figure 3**). Indeed low level of ROS is important for normal osteoclastogenesis and osteoblastogenesis to sustain bone integrity, whereas high level of ROS may contribute to mitochondrial dysfunction and alterations in signaling pathways and gene expression that in turn cause chondrocyte apoptosis and senescence, subsequent cartilage degradation, and changes in subchondral bone and processes of bone resorption and remodeling.

ROS signaling and nuclear programing

Recently several studies have drawn a clear link between ROS signaling and nuclear cell programming. At physiological levels, ROS can alter nuclear reprogramming of somatic cells to pluripotency, restricting cellular and genomic damage during self-renewal or preventing inappropriate differentiation of pluripotent cells.[103,104] The mechanism can involve the inhibition of Nox family members, which increases genomic stability and/or the activation of innate immunity as a positive feedback cycle of ROS generation.[105] During early reprogramming, antioxidant enzymes are upregulated, Nrf2 expression gradually increased in coordination with changes in mitochondrial biogenesis and bioenergetic functions.[105] Supplementary mechanism to affect pluripotency is the regulation of H_2O_2 production from superoxide and consequent cysteine oxidation of transcriptional factors or epigenetic modifiers by H_2O_2.[106] Such findings encourage the researchers to use inducible pluripotent stem cells (or adult stem cells) for the treatment of degenerative diseases like OA, where an alteration of the cartilage and bone tissue reparative capacity has been described. Experiments with undifferentiated human mesenchymal stem cells (hMSCs) have shown higher levels of glycolytic enzymes, increased rate of glycolysis for energy supply and of ROS signaling.[107] During the differentiation of hMSCs to osteoblast cells however, the ability to produce ROS is lost because of increased ROS scavenging and upregulation of antioxidant enzymes, and of transition from glycolysis to oxidative phosphorylation.[108] The underlying mechanism involves initial increase of Nrf2, NF-κB and AP-1 activity, and induction of hypoxia-inducible factor expression (HIF-α).[108]

ROS signaling and OA synovial fibroblasts

Increased expression and protein content of the pro-oxidant enzymes Nox2 and XO have been shown in synovial tissue of patients with knee OA.[109] The level of the enzymes

correlates with local expression of prolidase, an imidodipeptidase with a role in the recycling of collagen.[110] Prolidase is regulated at the post-transcriptional level by integrin-dependent signaling delivered from stimulatory (*i.e.*, thrombin) or inhibitory (*i.e.*, echistatin) β_1-integrin ligands or by nitric oxide donors.[110] Indeed in serum of patients with knee OA, increased oxidant (measured by ROS, total peroxide and lipid hydroperoxide levels) and decreased antioxidant state (measured by antioxidant capacity, thiol level and catalase enzyme activity) are related with elevated serum prolidase activity[111] and elevated inflammasome activity in synovium, indicating a link between ROS signaling, inflammation and cartilage collagen metabolism.[109]

OA synovial fibroblasts show higher production of chemokines, IL-1β, TNF-α and increased expression of thrombin.[112,113] Thrombin contributes to fibrin deposition, angiogenesis, proliferation and proinflammatory processes in OA synovium involving the intensive cleavage of osteopontin[114] and the activation of HO-1.[113] The latter enzyme is a stress-inducible rate-limiting enzyme in heme degradation responsible for cytoprotection against oxidative injury and is upregulated via the activation of PKCδ, c-Src, and Nrf2.[113] Thrombin secreted by OA fibroblasts can also alter the metabolism of cartilage chondrocytes via HO-1. The mechanism involves an increased production of insulin-like growth factor-1, a reduction of insulin-like growth factor binding protein-3, a decreased activation of ERK 1/2, an elevated activity of MMPs.[115] Alterations in OA synovial fibroblasts have been also related to their increased sensitivity to local NO donors which directly contribute to elevated ROS production and oxidative stress.[116] Oxidative stress in OA fibroblasts trigger MAPKs phosphorylation, TAK1-mediated NF-kB activation and Cox-2/PGE2 expression, both reversible by antioxidants such as N-acetylcysteine and hyaluronic acid.[116] The production of local inflammatory mediators and ROS in OA synovium sustains the proliferative ability of OA fibroblasts and alters the regulatory mechanisms of MMPs, cytokine and growth factors expression. For example IL-1β as well as ROS can induce the expression of particular miRNAs, which control NF-kB activation, translocation and acetylation. On the other hand, miRNA expression can be controlled by histone deacetylases (HDACs), enzymes sensitive to ROS signaling. Recent studies have shown that HDAC inhibitors can directly suppress gene expression of iNOS and MMPs in OA fibroblasts. Indirect effects of their administration include the alteration of NF-κB activity and translocation, changes in phosphorylation of p38 and ERK, and control of miR-146a expression.[117,118]

ROS signaling and OA chondrocytes

Articular human normal cartilage is composed of a hydrated extensive extracellular matrix (ECM) in which a small number of cells (2-3% of the total tissue volume) named chondrocytes are embedded. These cells are the only cell type in the normal mature articular cartilage, which are metabolically very active and normally do not divide after adolescence. They maintain homeostasis and balance of matrix synthesis and degradation, as well as repair damaged cartilage tissue in OA.[10,119] Cartilage catabolism comprises secretion of proteases, suppression of matrix synthesis and inhibition of chondrocyte proliferation, while the anabolism is associated with secretion of antagonistic cytokines, synthesis of protease inhibitors, production of extracellular matrix and cell replication. Early changes in the articular surface include fragmentation, fibrillation and gradual thinning of cartilage and loss of viscosity of the synovial fluid with alteration in the biochemical composition.[10,119]

Articular chondrocytes from OA patients are deteriorated and have irregularly orientated mitochondria. Indeed chondrocytes near the fibrillated regions show swollen mitochondria with disoriented cristae and nearby glycogen vacuoles, indicative of altered cell metabolism.[120] In culture, OA chondrocytes show increased mitochondrial mass [as a result of increased citrate synthase (CS) activity] but reduced mitochondrial respiratory activity and changes in mitochondrial membrane potential in comparison to normal chondrocytes.[14,121] The collapse of the mitochondrial membrane potential results in mitochondrial swelling, disruption of outer mitochondrial membrane and release of pro-apoptotic factors, such as cytochrome c, apoptosis-inducing factor and pro-caspases.[14] A proteomic study has demonstrated that enzymes involved in glycolysis significantly decrease in OA chondrocytes[119] along with a deficiency in mitochondrial SOD, catalase and glutathione scavenging systems; overproduction of ROS, NO, detrimental peroxynitrite and reduction of the ATP pool.[119] Several genomic, lipidomic[122] and high-resolution mass spectrometry analysis[123] have identified enrichment in pathways related to lipid (eicosanoids) metabolism and oxidative stress in OA chondrocytes. It is assumed that excessive cartilage load can trigger abnormalities in ROS release, mitochondrial functions and energy/metabolic state in some predisposed individuals and in some areas of cartilage. In those individuals altered ROS signaling can be sustained by aging, itself, resulting in the senescence secretory phenotype of chondrocytes. This phenotype includes the upregulation of the expression of matrix metalloproteinases (MMPs) and aggrecanases, cytokines and growth factors and the integration with the PI3K-Akt, p38, ERK and NF-kB signaling linking cell receptor with metabolic pathways.[124] Consequently, more chondrocytes at the

superficial zone acquire alterations in total DNA methylation profile,[125] in histone acetylation of anabolic genes (like SOX9 gene) and in particular miRNA leading to a failure in regulation of transcription and stability of antioxidant, inflammatory and metabolic genes.[126] The persistent ROS generation in combination with altered ROS scavenging in cartilage cause a progressive mitochondrial damage in most of the chondrocytes with changes in oxidative phosphorylation, redox signaling, miRNAs-regulated mitochondrial biogenesis[127] and expression of mitochondrial ROS antagonist chaperones.[119] As a result, chondrocyte apoptosis at superficial cartilage areas increases, and chondrocytes at deeper zone become hypertrophic disrupting normal endochondral ossification and matrix repair. Hypertrophic chondrocytes usually have metabolic and proliferation defects that can be overcome by the antioxidant treatment with N-acetylcysteine[128] or the administration of 17β-estradiol.[129] They also show alteration in HO-1. The enzyme is responsible for the degradation of heme to carbon monoxide (CO), free iron and biliverdin-IX. In mammals, biliverdin-IX is further converted to bilirubin-IX, and endogenous radical scavenger with recognized anti-inflammatory properties. On the other hand free iron is rapidly sequestered into the iron-storage protein ferritin, leading to additional antioxidant and antiapoptotic effects. CO has also several biological functions, with antiapoptotic and anti-inflammatory properties.[113]

Recently it has been shown that the accumulation of ROS in chondrocytes can lead to mitochondrial DNA (mtDNA) mutations. Researchers have even identified the specific mitochondrial DNA haplogroups J and T that have been associated with variations in the prevalence and progression of cartilage loss in some European populations.[130-133] In particular, J haplotype has been linked to lower serum levels of markers of collagen type-II degradation and of matrix metalloproteinases and higher ATP levels in the Spanish population, while T haplotype correlates with lower disease risk in a small UK cohort.[131-133]

ROS signaling and OA osteoclasts and osteoblasts

The osteoclasts drive the processes of bone resorption and bone matrix loss. The osteoclasts are differentiated from bone marrow monocyte precursors. The process is dependent on NF-kB signaling driven by RANKL-RANK or is regulated by IL-17, GM-CSF and/or IFN-γ. Osteoblasts, stem cells, stromal cells, neutrophils, NK cells and even migrated in the bone marrow $CD4^+$ T cells can drive osteoclastogenesis via direct contact. It has been shown that RANKL-RANK system stimulates ROS generation in osteoclasts.[134] The administration of the antioxidants N-acetylcysteine and glutathione attenuates RANKL-mediated Akt and

ERK activation and NF-kB signaling (via inhibition of IKK activity and IkBa phosphorylation) as well as it reduces bone resorbing activity and regulates osteoclasts apoptosis.[135] RANKL-mediated ROS production and osteoclast differentiation is abrogated and blocked when bone marrow precursors are depleted of Nox1 by siRNA or by expression of a dominant-negative mutant of Rac1.[134] The same group has shown that the role of ROS signaling for osteoclast differentiation is dependent on activation of JNK The osteoblasts drive the opposite process of bone resorption, bone remodeling and repair. In OA the balance between activation of osteoclasts and osteoblasts leads to abnormal remodeling and formation of osteophytes. Similarly to osteoclastsand p38 and transcription factor NF-ATc1.[134] RANKL can attenuate Nox2 mRNA expression but it can upregulate Nox1 and Nox3 transcription during the differentiation of macrophage linage to osteoclasts.[136] Indeed siRNA targeting of p67(phox) or p22(phox) in macrophage linage downregulates ROS generation, suppresses RANKL-induced osteoclastogenesis, inhibits the expression of osteoclast marker genes (TRAP, cathepsin K, Atp6i, ClC-7, and NF-ATc1).[136]

Osteoblasts drive the opposite process of bone resorption, bone remodeling and repair. In OA the balance between activation of osteoclasts and osteoblast leads to abnormal remodeling and formation of osteophytes. Similarly, to osteoclasts, osteoblasts are sensitive to ROS signaling during their differentiation from mesenchymal cells. Osteoblastogenesis involves Runx2-dependent and -independent mechanisms of gene expression and is dependent on growth factor signaling pathways triggered by bone morphogeneic proteins (BMPs), transforming factor (TGF)-β and Wnt cascade. Recently it has been shown that BMP-2 induces a rapid generation of ROS and activation of NADPH oxidase and that Nox4 activation is a key factor for osteoblast differentiation in response to BMP-2.[137] In differentiated OA osteoblasts however oxidative stress can have several impacts on their functions. The administration of the antioxidant N-acetylcysteine elevates osteocalcin and type I collagen synthesis and decreases alkaline phosphatase activity in OA osteoblasts. The mechanisms involve activation of p38 and JNK1/2, but not ERK1/2 kinases, induction of ATF-2/CREB activation, inhibition of NF-κB, and activation of COX-2 promoter activity via CRE site. Consequently, the antioxidant increases COX-2 and PGE_2 levels but decreases TNF-α induced IL-6 production.[138]

Nrf2 and OA pathogenesis

As a master regulator of the cellular redox homeostasis, Nrf2 induction may play a key role in OA pathophysiology. Nrf2 is responsible for the activation of glutathione-S-transferase, NQO1,[12] and glutathione peroxidase activities[139] thus attenuating early joint pain.[140] Nrf2 is a promoter of HO-1 expression,[102,141] prevents 4-hydroxynonenal (HNE)-induced mitochondrial damage,[3] and its deficiency in mice leads to more severe OA development.[141] HO-1 and γ-glutamylcysteine ligase (γ-GCLC) act synergistically as phase 2 antioxidants and were found in higher levels due to Nrf2 activation.[142] In addition, Nrf2 is involved in the regulation of ROS production by mitochondria and NADPH oxidase.[143]

Studies in Nrf2-deficient mice have shown that they are highly susceptible to chemical-induced toxicity and oxidative burden, as a result of dysregulation/loss of expression of detoxification, xenobiotic metabolism and redox genes.[144,145] However other genes including those involved in protein transport, ubiquitination, phosphorylation, cell cycle, growth and apoptosis have been identified to be also Nrf2-dependant.[146] By using ChIP-Seq and microarray analyses, 645 basal and 654 inducible direct targets of Nrf2 were identified and confirmed to be related with stress response and cell proliferation in mouse embryonic fibroblasts.[147] In humans, polymorphisms in the ARE sequences, to which Nrf2 binds, have been reported.[148] Concerning inflammatory responses, a recent review discussed the role of Nrf2 in the regulation of the expression of HO-1 during hematopoiesis, development and activation of lymphoid cells and macrophages as well the interaction between Nrf2 and NF-kB and the mechanism of Nrf2-regulated NLP3 inflammasomal activity.[149]

Abnormal ROS signaling in OA synovial fibroblasts by cytokines, thrombin or stress is associated with increased Nrf2 activity. Oxidative stress in cartilage may trigger activation of Nox4 and mitochondrial dysfunction. However, both factors may be counteracted by increased Nrf2 activation in fibroblasts.[150] Nrf2 regulates thrombin-induced production of HO-1 in synovial fibroblasts and consequently, inhibition of the Nrf2-dependent signaling pathway by Nrf2 siRNA, diminishes HO-1 expression. Thrombin increases the Nrf2 phosphorylation and binding to the ARE element of HO-1 gene promoter. Nrf2 activity is attenuated by inhibitors of PAR1/PAR3, PKCδ, and c-Src pathways indicating that all of them are involved in thrombin-induced Nrf2 activation in human synovial fibroblasts.[113] Nrf2 activation antagonizes cytokine-induced NF-kB signaling by limiting IkBα degradation, suggesting that a failure in this reciprocal interaction may also occur in OA synovial fibroblasts as they over-produce inflammatory cytokines.[149] However, the specific environment in OA cartilage may contribute to a different role of Nrf2 in OA fibroblasts in

comparison to that in fibroblasts from other tissues. For example skin fibroblasts from patients with systemic sclerosis show decreased Nrf2 activity along with elevated inflammatory gene expression and fibrosis. In skin fibroblasts, the Nrf2 silencing constitutively elevates collagen synthesis, spontaneous myofibroblast differentiation, and enhances TGF-ß responses[151] showing that Nrf2 may be a key cell-intrinsic anti-fibrotic factor sustaining extracellular matrix homeostasis in systemic sclerosis.[151] In another study using human periodontal ligament fibroblasts, it has been demonstrated that H_2O_2-mediated oxidative stress decreases the osteogenic differentiation of fibroblasts via interference with Wnt/β-catenin signaling and Nrf2 expression at protein and mRNA levels.[152]

Nrf2 plays a major chondroprotective role in the progression of OA and suppresses IL-1β-induced MMP-1, MMP-3 and MMP-13 expression at both mRNA and protein levels, and induces the expression of phase 2 antioxidant enzymes, such as NQO1 and HO-1,[9,11,141] as well as suppresses the PGE2 and NO production and inhibition of the phosphorylation of NF-κB p65 and IκBa in human OA.[11] It has been demonstrated that histone deacetylase inhibitors (HDACi),[141] physical activity,[142] phytochemicals, such as protandim, 6-gingerol,[3] ascorbic acid,[153] sulforaphane,[154] curcumin,[155] carotenoids,[156] as well as broccoli[157] and sesame oil are protective in models of OA by activation of Nrf2.[139] Downregulation of Nrf2 could result in inhibition of chondrogenesis through apoptotic cell death, but on the other hand stable overexpression of Nrf2 caused inhibition of chondral differentiation by reduction of the characteristic markers, such as type II collagen (Coll II), type X collagen (Coll X) and osteopontin. This means that appropriate expression and fine balance in Nrf2 activity is absolutely necessary for normal chondrogenesis and proper regulation of OA.[12,23] Nrf2 can also regulate indirectly chondrocyte apoptosis and senescence via the expression of glyoxalase I (an enzyme that detoxifies methylglyoxal).[10] This enzyme regulates the levels of the advanced glycation products (AGEs).[10] The levels of AGE such as urinary pentosidine correlate with the degree of cartilage damage and chondrocyte loss.[158,159] Thus, the overexpression of the receptors of AGEs in OA might suppress Nrf2 and glyoxalase I, respectively.[10]

Nrf2 has been reported to play important roles in the regulation of inflammatory processes. The induced expression of IL-1β and TNF-α results in higher expression of NO, which inhibits chondrocytes cytoskeletal actin polymerization and beta-1 integrin-dependent signaling, decreased expression of IL-1β receptor antagonist and TGF-β, suppression of chondrocyte collagen and proteoglycan synthesis, activation of MMPs and suppression of proliferation, as well as, promotion of apoptosis. NO suppresses energy metabolism and

directly induces calcification of articular cartilage. During apoptosis mitochondria sequester Ca^{2+} in providing energy to the cells. The excessive Ca^{2+} concentrations lead to the opening of the mitochondrial permeability, which depolarizes the mitochondria and leads to mitochondrial swelling and apoptosis.[14] Nrf2 inhibits NF-kB activation and the production of inflammatory mediators, such as IL-1β and PGE2, as well as, MMP-1, MMP-3 and MMP-13, which are important risk factors that have the ability to inhibit type II collagen synthesis in ECM.[11,154] The mechanisms of this inhibition are just beginning to be understood, and include repression of transcription of pro-inflammatory genes.[160]

Several lines of evidences indicate that Nrf2 is an important factor for osteoclast and osteoblast differentiation as well as to sustain normal skeletal and bone metabolism. Nrf2-deficient mice have lower bone mass and bone strength.[161] The same authors described that the deletion of Nrf2 inhibits the load-driven gene expression of antioxidant enzymes and Wnt5a in cultured primary osteoblasts[161] indicating that Nrf2 regulates the overall bone homeostasis. Nrf2 is a key factor in the maintenance of bone microarchitecture as observed in an animal model of postmenopausal osteoporosis.[162] Nrf2 deficiency promotes the RANKL-induced activation of p38, c-Jun, ERK, induces c-Fos and NF-kB activation and osteoclastogenesis, likely due to dysfunction in the production of antioxidant enzymes and glutathione.[163] The overexpression of Nrf2 enhances the expression of anti-oxidant enzymes, inhibits osteoclast differentiation and attenuates lipopolysaccharide-mediated RANKL-dependent cranial bone destruction *in vivo*.[164] The same authors showed that during osteoclastogenesis, the precursor cells lose Nrf2, fail to translocate Nrf2 in the nucleus because of Keap1 upregulation, and downregulate cytoprotective enzymes (HO-1, γ-glutamylcysteine synthetase, and glucose-6-phosphate dehydrogenase), elevating ROS production.[164] Consistent with this report is a study demonstrating increased oxidative stress in bone marrow-derived cells from ovariectomized Nrf2-deficient mice and a higher responsiveness of bone marrow-derived cells to osteoclastogenic stimuli in vitro.[162] Knockout mice of BTB and CNC homology 1 (Bach1)—the competitor of Nrf2 in transcriptional regulation attenuate RANKL-mediated osteoclastogenesis.[165] The mechanism involves RANKL-dependent nuclear import of Bach1 and Nrf2 nuclear export, which attenuates Nrf2-mediated antioxidant enzymes and ROS signaling in osteoclasts. Moreover, the induction of Bach1 nuclear export inhibited bone destruction *in vivo*.[165] Nrf2 knockdown or deletion increases osteoclastic differentiation from bone marrow-derived macrophages via upregulation of transcription factor NFATc1.[166] Interestingly, in this study Nrf2 inhibits osteoblast differentiation and mineralization via suppression of Runx2, osteocalcin, and

osterix.[166] The number of osteoblasts in bone is increased in Nrf2-knockout mice in comparison to wild-type mice, indicating that both osteoclastogenesis and osteoblastogenesis are dependent of Nrf2 or at least require its presence.[166] When Nrf2-deficient mice are irradiated, the bone loss and RANKL expression markedly increase, but the mineralization of calvarial osteoblasts decreases, suggesting that loss of Nrf2 can activate ROS signaling, altering the sensitivity to radiation-induced bone damage. Importantly, all of these effects are restored by the antioxidant N-acetylcysteine.[167] The role of Nrf2 in osteoblast-driven bone formation has been demonstrated in a model of fracture healing. The underlying mechanism is related to elevated expression of vascular endothelial growth factor (VEGF) and diminished production of osteocalcin in Nrf2-deficient mice.[168] Together, these studies have shown that Nrf2 plays a key role in regulation of the balance between osteoclast-driven bone resorption and osteoblast-driven remodeling (**Figure 4**).

However, in Nrf2-deficient mice, altered bone metabolism has not been studied with respect to a dysfunctional immunity or/and cytokine production imposing the need for further investigations. It has not been yet considered whether Nrf2 has a critical role in maintaining hematopoietic stem cell function, including effects on cell quiescence and self-renewal potential. Nrf2 favors the commitment of both myeloid and lymphoid lineages suggesting that Nrf2 imposes a general regulatory role in bone marrow niches.[169] Thus, Nrf2 may regulate osteoclastogenesis or osteblastogenesis by stabilization of bone marrow niches with progenitors. It has been shown that Nrf2-deficient mice have more severe cartilage damage in two models of OA, destabilization of the medial meniscus (DMM) and monosodium iodoacetate (MIA) models.[141] The chondroprotective role of Nrf2 in these models was mediated by the use of HDAC inhibitors inhibiting OA-associated proteins MMP1, 3, 13 and pro-inflammatory cytokines TNF-α, IL-1β, and IL-6. Because inhibition of HDAC fails to improve the disease in Nrf2-deficient mice,[141] this supports the notion for a more general cell dysfunction that is likely mediated by epigenetic mechanisms.

The manipulation of stem cells via Nrf2

The manipulation of stem cells (from different origin; stem cells or adipose-driven) is a new strategy exploiting the key role of Nrf2 in cell differentiation, senescence and apoptosis. It has been shown that the generation of osteoblast cells from stem cells can be achieved by silencing of Nrf2 and autophagy. *In vivo*, bone formation is enhanced by injection of Nrf2 knockdown stem cells.[170] Moreover, therapy with adipose-driven stem cells can be improved

by promoting endothelial progenitor cell migration and angiogenesis under hypoxia via TLR4-mediated NF-kB signaling and antioxidant-driven Nrf2 activation.[171]

Anti-inflammatory drugs in OA therapy

Currently, there is no enough satisfactory drug for effective OA treatment and hence OA cannot be cured totally. Moreover, the existing treatment strategies aim only to control the symptom and reduce the pain.[22] Although there are promising candidates they are limited in efficacy and associated with certain toxicity.[70] The current management of OA includes: first, behavioral interventions (patient education, exercise and change in life style); second, simple analgesic, such as acetaminophen (paracetamol); third, application of nonsteroidal anti-inflammatory drugs (*e.g.*, COX-2 inhibitors); fourth, intraarticular injections of corticosteroids, hyaluronic acid and glucosamine; and fifth, total joint replacement in the most severe cases of OA.[22,70] Bisphosphonates might have symptomatic and structural benefits for patients with OA. Pamidronate disodium was able to prevent completely OA pathology in rabbits through osteoprotegerin receptor activator of NF-kB ligand. Aldendronate reduced osteophyte formation and articular cartilage degeneration.[9] HDACi have emerged as potential therapeutic strategy of OA treatment. Its effect is linked with the acetylation of Nrf2, which enhances its functions in OA.[141]

Effect of plant derived or synthetic antioxidants on Nrf2 expression in osteoarthritis

Plants-derived antioxidants have a great potential to modulate Nrf2 expression. For example, curcumin has well proven anti-oxidant activity and can protect human chondrocytes from apoptosis and dysfunction by inhibiting AP-1/NF-kB signalling and consequent production of IL-1β and activation of MMP-3.[172] The inhibitory effect on NF-kB signaling by curcumin may be, at least in part, due to the activation of Nrf2. Curcumin stimulates HO-1 gene activity by promoting inactivation of the Nrf2–Keap1 complex, leading to increased Nrf2 binding to the resident HO-1 AREs.[173] Calycosin, a compound derived from the Chinese medicinal herb *Radix Astragali*, has similar effect as it activates HO-1 and Nrf2 in synovial fibroblasts from arthritic patients, and inhibits pro-inflammatory factors.[174] The widely applied antioxidant ascorbic acid (AA) was able to reduce apoptosis and loss of viability, and markedly decreased H_2O_2-mediated senescence of chondrocytes. AA stimulated the expression of collagens and proteoglycans, as well as inhibited chondrocyte differentiation under oxidative stress through

decreasing the activity of Nrf2, NF-kB, AP1 and MMP-3.[153] Sesame oil treatment for seven days significantly decreased OA-associated joint pain, by decreasing IL-6, increasing CS activity and myosin heavy chain IIa mRNA expression. Sesame oil decreased muscular lipid peroxidation, activated Nrf2, inhibited ROS and increased glutathione production and glutathione peroxidase activity.[139] Protandim® (plant-derived science-based formulation) and 6-gingerol preserved chondrocytes viability and mitochondrial metabolism through Nrf2 upregulation and subsequent reduction of IL-1β and MMP-13.[3] Wogonin, a naturally occurring flavonoid, revealed potent anti-inflammatory and chondroprotective effects through the activation of ROS/ERK/Nrf2 signaling pathways in human osteoarthritis chondrocytes. This molecule completely suppressed the expression and production of inflammatory mediators (IL-6, COX-2, PGE2, iNOS and NO), as well as, matrix degrading proteases including MMP-13, MMP-3, MMP-9.[175]

It is noteworthy that the dose responses of exposures to some of the phytochemicals discussed above are often non-linear J-shaped or inverted U-shaped.[176,177] Dose responses of this type are known as hormetic dose responses, and are characterized by a low-dose stimulation and a high-dose inhibition.[178] The initial event is mild stress, which subsequently engages cellular stress response mechanisms, including those regulated by Nrf2. The transcriptional upregulation of Nrf2-dependent as well as other inducible cytoprotective proteins (also known as vitagenes) allows adaptation and survival, and enhanced resistance to subsequent challenges, such as those caused by oxidative stress and chronic inflammation.[179]

In addition, natural and synthetic compounds can modulate NLRP3 inflammasome activation via Nrf2. For example treatment of mice with lupus nephritis with epigallocatechin-3-gallate (EGCG) or with citral (3,7-dimethyl-2,6-octadienal; a major active compound in a Chinese herbal medicine *Litsea cubeba*) attenuates NLRP3 inflammasome activation via Nrf2 signaling.[180,181] By a similar mechanism, mangiferin (2-C-β-d-gluco-pyranosyl-1,3,6,7-tetrahydroxyxanthone) protects mice with sepsis-induced acute kidney-injury,[182] biochanin inhibits acute liver injury,[183] and antroquinonol (active compound from *Antrodia camphorata)* mitigates IgA nephropathy in mice.[184]

Several studies have pointed to the cytoprotective potential of Nrf2 against glucocorticoid-induced apoptosis of osteoblastic cells. Dexamethasone-induced ROS production is suppressed by Nrf2 activation and antioxidant enzymes expression by indole-3-carbinol[185] and by sulforaphane (a naturally occurring isothiocyanate)[186] and icariside II.[187] Notably, sulforaphane, one of the most potent naturally occurring Nrf2 activators known to date, has been shown to suppress pro-inflammatory resposnes and protect cultured

human chondrocytes against various pro-inflammatory and pro-apoptotic stimuli, including shear stress.[188,189] When included in the diet, sulforaphane (3 μM/day) decreased the arthritis score in a mouse model of osteoarthritis.[154] The cytotoxic effect of methylglyoxal on osteoblastic cells was reversed by glabridin by Nrf2 activation, increases in HO-1 and glyoxalase I, and normalization of mitochondrial function.[190] Icariside II, synthetic SC79 and OSU53 can also prevent dexamethasone-induced apoptosis of osteoblasts via Akt activation downstream of Nrf2.[187,191,192] All of these compounds may have potential to limit osteoblast cell death, which could lead to osteoporosis or osteonecrosis.

Conclusions and future directions

ROS signaling may play a dual role in OA. At low physiological levels, ROS can be important for cartilage and bone integrity by sustaining nuclear programing during chondrogenesis, osteoclasts and osteoblasts differentiation probably via Nrf2-sequestration mechanisms. Aging, mechanical load and inflammation can increase ROS production and can trigger oxidative stress with mitochondrial dysfunction, disturbances in cell signaling and alterations in epigenetic control of gene expression. Consequently, high level of ROS signaling and altered Nrf2 activity can increase chondrocyte apoptosis with cartilage degradation, induce chondrocyte hypertrophy and dysfunction in subchondral bone. Decreased Nrf2 activity may be a result of a failure in its homeostatic post-translational regulation and/or altered epigenetic and transcriptional regulatory mechanisms. Experimental evidence suggests that the role of Nrf2 in bone integrity is complex. Low Nrf2 activity may sustain bone resorption favoring osteoclastogenesis at early stages of OA, whereas high Nrf2 activity may enhance osteoblastogenesis in advanced disease stage, inducing extensive bone remodeling. Therapeutic approaches to increase Nrf2 activity by pharmacological agents may counteract oxidative stress and inflammation in OA thus effectively limiting cartilage degradation and bone resorption, whereas overall restoration of homeostatic Nrf2 activity may be required to normalize imbalanced bone resorption and remodeling.

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Conflict of interest

The authors declare no conflicts of interest.

Figure Legends

Figure 1. Domain structure of Nrf2. The relative positions of the Nrf2 ECH homology (Neh) 1–7 domains are shown. The numbering of amino acids is based on the human protein. The DLG and ETGE motifs in the Neh2 domain that bind Keap1, and the DSGIS and DSAPGS motifs in the Neh6 domain that bind β-TrCP, are indicated. The Neh4 and Neh5 domains form the transactivation domain of Nrf2 that recruits CREB-binding protein (CBP). The retinoid X receptor α (RXRα) binds to the Neh7 domain of Nrf2, leading to repression of the transcription factor. Neh1 mediates binding to DNA and to the heterodimeric partner of Nrf2, a small Maf transcription factor. Neh3 recruits the chromo-ATPase/helicase DNA-binding protein 6 (CHD6).

Figure 2. Regulation of Nrf2 by ubiquitination and proteasomal degradation. Three known ubiquitin ligase systems mediate the ubiquitination of Nrf2: (i) Kelch-like ECH associated protein 1 (Keap1), a substrate adaptor protein for Cullin-3 (Cul3)-Rbx1/Roc1 ubiquitin ligase (i.e. CRL^{Keap1}), which binds through its Kelch domains to the DLG and ETGE motifs in the Neh2 domain of Nrf2. For substrate adaptor activity, the cysteine sensors of Keap1 are in reduced state; (ii) β-transducin repeat-containing protein (β-TrCP), a substrate adaptor for the Skp1-Cul1-Rbx1/Roc1 E3 ligase complex (i.e. $SCF^{\beta\text{-}TrCP}$). Recognition by β-TrCP requires formation of a DSGIS motif-containing phosphodegron in the Neh6 domain of Nrf2 by glycogen synthase kinase 3 (GSK3), which in turn requires phosphorylation of Nrf2 by a priming kinase; and (iii) the E3 ubiquitin ligase Hrd1/synoviolin, an endoplasmic reticulum (ER) protein. Degradation by Hrd1 has been shown to occur during ER stress.

Figure 3. Role of ROS signaling in OA pathology. The low level of ROS can sustain cartilage and bone integrity as ROS regulate nuclear programing during osteoclasts and osteoblasts differentiation. At high level ROS can trigger oxidative stress with mitochondrial dysfunction and a failure in cells signaling and epigenetic regulation of gene expression. Oxidative stress causes inflammation, chondrocyte apoptosis with cartilage degradation and dysfunction in subchondral bone with imbalanced bone resorption and remodeling.

Figure 4. Role of Nrf2 in OA. Bone resorption at early stage of OA is due to a favorable osteoclastogenesis via low Nrf2/Keap ratio (as shown by Nrf2 deficiency). In advanced stage of OA, intensive bone remodeling triggered as a compensatory mechanism to bone resorption is more likely related with enhanced osteoblastogenesis via high Nrf2/Keap ratio (as shown by Nrf2 overexpression). The therapy approaches in OA may involve a normalization of bone/resorption and remodeling via interference with Nrf2 expression and ROS signaling.

References:

1. Glyn-Jones, S., A. Palmer., R. Agricola, *et al*. 2015. Osteoarthritis. *The Lancet.* 386(9991): 376-387.
2. Wang, Y., A. Teichtahl, & F. Cicuttini, 2016. Osteoarthritis year in review 2015: imaging. *Osteoarthr. Cartilage*. 24(1): 49-57.
3. Abusarah, J., H. Benabdoune, Q. Shi, *et al.* 2016 Elucidating the role of protandim and 6-gingerol in protection against osteoarthritis. *J. Cell Biochem.* 9999: 1-11.
4. Davidson, R., O. Jupp, Y. Bao, *et al.* 2016. Can sulforaphane prevent the onset or slow the progression of osteoarthritis? *Nutr. Bull.* 41: 175-179.
5. Portal-Núñez, S., P. Esbrit, J. Alcaraz, *et al*. 2016. Oxidative stress, autophagy, epigenetic changes and regulation by miRNAs as potential therapeutic targets in osteoarthritis. *Biochem. Pharmacol.* 108: 1-10.
6. Musumeci, G., P. Castrogiovanni, F. Trovato, *et al*. 2015. Biomarkers of chondrocyte apoptosis and autophagy in osteoarthritis. *Int. J. Mol. Sci.* 16(9): 20560-20575.
7. Castañeda, S., J. Roman-Blas, R. Largo, *et al*. 2014. Osteoarthritis: A progressive disease with changing phenotypes. *Rheumatology (Oxford)*. 53(1): 1-3.
8. Sandell, L. 2012. Etiology of osteoarthritis: genetics and synovial joint development. *Nat. Rev. Rheumatol.* 8(2): 77-89.
9. Poulet, B. & K. Stainess. 2016. New developments in osteoarthritis and cartilage biology. *Curr. Opin. Pharmacol.* 28: 8-13.
10. Ahmed, U., P. Thornalley & N. Rabbani. 2014. Possible role of methylglyoxal and glyoxalase in arthritis. *Biochem. Soc. Trans*. 42: 538-542.
11. Kong, P., G. Chen, A. Jiang, *et al.* 2016. Sesamin inhibits IL-1β-stimulated inflammatory response in human osteoarthritis chondrocytes by activating Nrf2 signaling pathway. *Oncotarget.* 7: 83720-83726.
12. Hinoi, E., T. Takarada, S. Fujimori, *et al*. 2007. Nuclear factor E2 p45-related factor 2 negatively regulates chondrogenesis. *Bone.* 40: 337-344.
13. Gavriilidis, C., S. Miwa, T. von Zglinicki, *et al*. 2013. Mitochondrial dysfunction in osteoarthritis is associated with down-regulation of superoxide dismutase 2. *Arthritis Rheum.* 65: 378-387.
14. Blanco F, M. López-Armada & E. Maneiro. 2004. Mitochondrial dysfunction in osteoarthritis. *Mitochondrion.* 4: 715-728.

15. Kensler, T, N. Wakabayashi & S. Biswal. 2007. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu. Rev. Pharmacol. Toxicol.* 47: 89-116.
16. Pajares, M., A. Cuadrado & A. Rojo. 2017. Modulation of proteostasis by transcription factor NRF2 and impact in neurodegenerative diseases. *Redox Biology* 11: 543-553.
17. Hayes, J. & A. T. Dinkova-Kostova. 2014. The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. *Trends Biochem. Sci.* 39(4):199-218.
18. Mitsuishi, Y., K. Taguchi, Y. Kawatani, *et al*. 2012. Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming. *Cancer Cell.* 22(1): 66-79.
19. DeNicola, G., P. Chen, E. Mullarky, *et al.* 2015. NRF2 regulates serine biosynthesis in non-small cell lung cancer. *Nat. Genet.* 47(12): 1475-1481.
20. Holmström, K., L. Baird, Y. Zhang, *et al*. 2013. Nrf2 impacts cellular bioenergetics by controlling substrate availability for mitochondrial respiration. *Biology open* 2(8): 761-770.
21. Ludtmann, M., P. Angelova, Y. Zhang, *et al*. 2014. Nrf2 affects the efficiency of mitochondrial fatty acid oxidation. *Biochem. J.* 457(3): 415-424.
22. Li, Y., W. Xiao & W. Luo. 2016. Cellular aging towards osteoarthritis. *Mech. Ageing Dev.* DOI:10.1016/j.mad.2016.12.012.
23. Solomon, L., N. Bérubé & F. Beier. 2008. Transcriptional regulators of chondrocyte hypertrophy. *Birth Defects Res.* C 84: 123-130.
24. McMahon, M., N. Thomas, K. Itoh, *et al*. 2004. Redox-regulated turnover of Nrf2 is determined by at least two separate protein domains, the redox-sensitive Neh2 degron and the redox-insensitive Neh6 degron. *J. Biol. Chem.* 279(30): 31556-31567.
25. Itoh, K., N. Wakabayashi, Y. Katoh, *et al*. 1999. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* 13(1): 76-86.
26. Kobayashi, A., I. Kang, H. Okawa, *et al*. 2004. Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Mol. Cell. Biol.* 24(16): 7130-7139.

27. Dinkova-Kostova A.T., R. Kostov & P. Canning. 2016. Keap1, the cysteine-based mammalian intracellular sensor for electrophiles and oxidants. *Arch. Biochem. Biophys.* 617: 84-93.
28. Chauhan, N., L. Chaunsali, P. Deshmukh, *et al*. 2013. Analysis of dimerization of BTB-IVR domains of Keap1 and its interaction with Cul3, by molecular modeling. *Bioinformation.* 9(9): 450-455.
29. McMahon, M., N. Thomas, K. Itoh, *et al*. 2006. Dimerization of substrate adaptors can facilitate cullin-mediated ubiquitylation of proteins by a "tethering" mechanism: a two-site interaction model for the Nrf2-Keap1 complex. *J. Biol. Chem.* 281(34): 24756-24768.
30. Tong, I., B. Padmanabhan, A. Kobayashi, *et al*. 2007. Different electrostatic potentials define ETGE and DLG motifs as hinge and latch in oxidative stress response. *Mol. Cell Biol.* 27(21): 7511-7521.
31. Tong, K, Y. Katoh, H, Kusunoki, *et al*. 2006. Keap1 recruits Neh2 through binding to ETGE and DLG motifs: characterization of the two-site molecular recognition model. *Mol. Cell Biol.* 26(8): 2887-2900.
32. Baird, L., D. Lleres, S. Swift, *et al*. 2013 Regulatory flexibility in the Nrf2-mediated stress response is conferred by conformational cycling of the Keap1-Nrf2 protein complex. *Proc. Natl. Acad. Sci. U S A.* 110(38): 15259-15264.
33. Tao, S., L. Pengfei, L. Gang, *et al*. 2017. p97 negatively regulates NRF2 by extracting ubiquitylated NRF2 from the KEAP1-CUL3 E3 complex. *Mol. Cell Biol.* DOI:10.1128/MCB.00660-16.
34. McMahon, M., D. Lamont, K. Beattie, *et al*. 2010. Keap1 perceives stress via three sensors for the endogenous signaling molecules nitric oxide, zinc, and alkenals. *Proc. Natl. Acad. Sci. USA.* 107(44): 18838-18843.
35. Zhang, D. & M. Hannink. 2003. Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Mol. Cell Biol*. 23(22): 8137-8151.
36. Dinkova-Kostova, A., W. Holtzclaw, R. Cole, *et al*. 2002. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc. Natl. Acad. Sci. U S A.* 99(18): 11908-11913.
37. Saito, R., T. Suzuki, K. Hiramoto, *et al*. 2015. Characterizations of three major cysteine sensors of Keap1 in stress response. *Mol. Cell Biol.* 36(2): 271-284.

38. Ma, Q. 2013. Role of nrf2 in oxidative stress and toxicity. *Annu. Rev. Pharmacol. Toxicol.* 53: 401-426.
39. Bertrand, H., M. Schaap, L. Bairdet *et al*. 2015. Design, synthesis, and evaluation of triazole derivatives that induce Nrf2 dependent gene ptoducts and inhibit the Keap1-Nrf2 protein-protein interaction. *J. Med. Chem.* 58(18): 7186-7194.
40. Abed, D., M. Goldstein, H. Albanyan, *et al*. 2015. Discovery of direct inhibitors of Keap1-Nrf2 protein-protein interaction as potential therapeutic and preventive agents. *Acta Pharm. Sin. B.* 5(4): 285-299.
41. Katsuoka F., H. Motohashi, T. Ishii, *et al*. 2005. Genetic evidence that small maf proteins are essential for the activation of antioxidant response element-dependent genes. *Mol. Cell. Biol.* 25(18): 8044-8051.
42. Nguyen, T., P. Sherratt & C. Pickett. 2003. Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. *Annu. Rev. Pharmacol. Toxicol.* 43: 233-260.
43. Hirotsu, Y., F. Katsuoka, R. Funayama, *et al*. 2012. Nrf2-MafG heterodimers contribute globally to antioxidant and metabolic networks. *Nucleic Acids Res.* 40(20): 10228-10239.
44. Lin, W., G. Shen, X. Yuan, *et al*. 2006. Regulation of Nrf2 transactivation domain activity by p160 RAC3/SRC3 and other nuclear co-regulators. *J. Biochem. Mol. Biol.* 39(3): 304-310.
45. He, H., P. Gong, B. Hu, *et al*. 2001. Identification of activating transcription factor 4 (ATF4) as an Nrf2-interacting protein. Implication for heme oxygenase-1 gene regulation. *J. Biol. Chem.* 276(24): 20858-20865.
46. Wang, H,, K. Liu, M. Geng, *et al*. 2013. RXRalpha inhibits the NRF2-ARE signaling pathway through a direct interaction with the Neh7 domain of NRF2. *Cancer Res.* 73(10): 3097-3108.
47. Sekine, H., K. Okazaki, N. Ota, *et al*. 2015. The mediator subunit MED16 transduces NRF2-activating signals into antioxidant gene expression. *Mol. Cell Biol.* 36(3): 407-420.
48. Malik, S. & R. Roeder. 2010. The metazoan mediator co-activator complex as an integrative hub for transcriptional regulation. *Nat. Rev. Genet.* 11(11): 761-772.
49. Allen, B. & D. Taatjes. 2015 The Mediator complex: a central integrator of transcription. *Nat. Rev. Mol. Cell Biol.* 16(3): 155-166.

50. Rada, P., A. Rojo, S. Chowdhry, *et al*. 2011. SCF/{beta}-TrCP promotes glycogen synthase kinase 3-dependent degradation of the Nrf2 transcription factor in a Keap1-independent manner. *Mol. Cell Biol.* 31(6): 1121-1133.

51. Chowdhry, S., Y. Zhang, M. McMahon, *et al*. 2013. Nrf2 is controlled by two distinct beta-TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity. *Oncogene* 32(32): 3765-3781.

52. Rojo, A., O. Medina-Campos, P. Rada, *et al*. 2012. Signaling pathways activated by the phytochemical nordihydroguaiaretic acid contribute to a Keap1-independent regulation of Nrf2 stability: Role of glycogen synthase kinase-3. *Free Radic. Biol. Med.* 52(2): 473-487.

53. Wu, T., F. Zhao, B. Gao, *et al*. 2014. Hrd1 suppresses Nrf2-mediated cellular protection during liver cirrhosis. *Genes Dev.* 28(7): 708-722.

54. Sun, Z., Y. Chin & D. Zhang. 2009. Acetylation of Nrf2 by p300/CBP augments promoter-specific DNA binding of Nrf2 during the antioxidant response. *Mol. Cell Biol.* 29(10): 2658-2672.

55. Miao, W., L. Hu, P. Scrivens, *et al*. 2005. Transcriptional regulation of NF-E2 p45-related factor (NRF2) expression by the aryl hydrocarbon receptor-xenobiotic response element signaling pathway: direct cross-talk between phase I and II drug-metabolizing enzymes. *J. Biol. Chem.* 280(21): 20340-20348.

56. Yeager, R., S. Reisman, L. Aleksunes, *et al*. 2009. Introducing the "TCDD-inducible AhR-Nrf2 gene battery". *Toxicol. Sci.* 111(2): 238-246.

57. Kang. H., Y. Hong, O. Rodriguez, *et al*. 2011. Detoxification: a novel function of BRCA1 in tumor suppression? *Toxicol. Sci.* 122(1): 26-37.

58. Sanderson, L., M. Boekschoten, B. Desvergne *et al*. 2010. Transcriptional profiling reveals divergent roles of PPARalpha and PPARbeta/delta in regulation of gene expression in mouse liver. *Physiol. Genomics* 41(1): 42-52.

59. DeNicola, G., F. Karreth, T. Humpton, *et al.* 2011. Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. *Nature* 475(7354): 106-109.

60. Kwak, M., K. Itoh, M. Yamamoto, *et al*. 2002. Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the nrf2 promoter. *Mol. Cell Biol.* 22(9): 2883-2892.

61. Yu, S., T. Khor, K. Cheung, *et al*. 2010. Nrf2 expression is regulated by epigenetic mechanisms in prostate cancer of TRAMP mice. *PLoS One* 5(1): e8579.
62. Yang, M., Y. Yao, G. Eades, *et al*. 2011. MiR-28 regulates Nrf2 expression through a Keap1-independent mechanism. *Breast Cancer Res. Treat.* 129(3): 983-991.
63. Singh, B., A. Ronghe, A. Chatterjee, *et al*. 2013. MicroRNA-93 regulates NRF2 expression and is associated with breast carcinogenesis. *Carcinogenesis*. 34(5): 1165-1172.
64. Wang, P., X. Liang, Y. Lu, *et al*. 2016. MicroRNA-93 downregulation ameliorates cerebral ischemic injury through the Nrf2/HO-1 defense pathway. *Neurochem. Res.* 41(10): 2627-2635.
65. Narasimhan, M., P. Dhyanesh, V. Dhanashree, *et al*. 2012. Identification of novel microRNAs in post-transcriptional control of Nrf2 expression and redox homeostasis in neuronal, SH-SY5Y cells. *PLoS One* 7(12): e51111.
66. Xue, W., X. Bai & L. Zhang. 2015. RhTNFR:Fc increases Nrf2 expression via miR-27a mediation to protect myocardium against sepsis injury. *Biochem. Biophys. Res. Commun.* 464(3): 855-861.
67. Sangokoya, C., M. Telen & J. Chi. 2010. MicroRNA miR-144 modulates oxidative stress tolerance and associates with anemia severity in sickle cell disease. *Blood*. 116(20): 4338-4348.
68. Zhou, S., W. Ye, Y. Zhang, *et al*. 2016. miR-144 reverses chemoresistance of hepatocellular carcinoma cell lines by targeting Nrf2-dependent antioxidant pathway. *Am. J. Transl. Res.* 8(7): 2992-3002.
69. Zhang, X., Ha, S, Wang, H. *et al*. 2015. Tanshinone IIA protects dopaminergic neurons against 6-hydroxydopamine-induced neurotoxicity through miR-153/NF-E2-related factor 2/antioxidant response element signaling pathway. *Neuroscience* 303: 489-502.
70. Goldring, M. & F. Berenbaum. 2015. Emerging tartgets in osteoarthritis therapy. *Curr Opin Pharmacol* 22: 51-63.
71. Sellam, J. & F. Berenbaum. 2010. The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. *Nat. Rev. Rheumatol*. 6(11): 625-635.
72. Bonin, C., E. Lewallen, S. Baheti *et al*. 2016. Identification of differentially methylated regions in new genes associated with knee osteoarthritis. *Gene*. 576: 312-318.

73. Benigni, G., P. Dimitrova, F. Antonangeli, *et al*. 2017. CXCR3/CXCL10 Axis regulates neutrophil–NK cell cross-talk determining the severity of experimental osteoarthritis. *J. Immunol.* 198(5): 2115-2124.

74. Ayral, X., E. Pickering, T. Woodworth, *et al.* 2005. Synovitis: A potential predictive factor of structural progression of medial tibiofemoral knee osteoarthritis - Results of a 1 year longitudinal arthroscopic study in 422 patients. *Osteoarthr. Cartil.* 13(5): 361-367.

75. Rushton, M., L. Reynard, M. Barter *et al*. 2014. Characterization of the cartilage DNA methylome in knee and hip osteoarthritis. *Arthritis Reum*. 66(9): 2450-2460.

76. den Hollander, W., Y. Ramos, N. Bomer, *et al*. 2015. Transcriptional associations of osteoarthritis-mediated loss of epigenetic control in articular cartilage. *Arthritis Rheum.* 67(8): 2108-2116.

77. Haseeb, A., M. Makki & T. Haqqi 2014. Modulation of ten-eleven translocation 1 (TET1), isocitrate dehydrogenase (IDH) expression, alpha-Ketoglutarate (alpha-KG), and DNA hydroxymethylation levels by interleukin-1beta in primary human chondrocytes. *J. Biol. Chem.* 289(10): 6877-6885.

78. Merz, D., R. Liu, K. Johnson, *et al*. 2003. IL-8/CXCL8 and Growth-Related Oncogene α/CXCL1 Induce Chondrocyte Hypertrophic Differentiation. *J. Immunol.* 171(8): 4406-4415.

79. Chauffier, K., M. Laiguillon, C. Bougault, *et al*. 2012. Induction of the chemokine IL-8/Kc by the articular cartilage: Possible influence on osteoarthritis. *Joint Bone Spine.* 79(6): 604-609.

80. Toncheva, A., M. Remichkova, K. Ikonomova, *et al*. 2009. Inflammatory response in patients with active and inactive osteoarthritis. *Rheumatol. Int.* 29(10): 197-1203.

81. Dimitrova, P., N. Ivanovska, L. Belenska, *et al*. 2012. Abrogated RANKL expression in properdin-deficient mice is associated with better outcome from collagen-antibody-induced arthritis. *Arthritis Res. Ther.* 14(4): R173.

82. Ivanovska, N., & P. Dimitrova. 2010. Bone resorption and remodeling in murine collagenase-induced osteoarthritis after administration of glucosamine. *Arthritis Res. Ther.* 13(2): R44.

83. Milanova, V., N. Ivanovska & P. Dimitrova. 2014. The effect of interleukin 17 and Toll-like receptor 2 on CD11b expression and apoptosis of neutrophils in zymosan-induced arthritis and paw oedema. *Cent. Eur. J. Immunol.* 39(2): 131-141.

84. Montecino-Rodriguez, E., B. Berent-Maoz & K. Dorshkind. 2013. Causes, consequences, and reversal of immune system aging. *J. Clin. Invest*. 123(3): 958-965.

85. Nathan, C. & A. Cunningham-Bussel. 2013. Beyond oxidative stress: an immunologist's guide to reactive oxygen species. *Nat. Rev. Immunol*. 13(5): 349-361.

86. Paulsen, C. & K. Carroll. 2010. Orchestrating redox signaling networks through regulatory cysteine switches. *ACS Chem. Biol.* 5(1): 47-62.

87. Nemoto, S. & T. Finkel. 2002. Redox regulation of forkhead proteins through a p66shc-dependent signaling pathway. *Science*. 295(5564): 2450-2452.

88. Lambeth, J. 2004. NOX enzymes and the biology of reactive oxygen. *Nat. Rev. Immunol*. 4(3): 181-189.

89. Chen, K., S. Craige & J. Keaney. 2009. Downstream targets and intracellular compartmentalization in Nox signaling. *Antioxid. Redox Signal.* 11(10): 2467-2480.

90. Wu, R., Y. Xu, Z. Ma, *et al*. 2005. Subcellular targeting of oxidants during endothelial cell migration. *J. Cell Biol*. 171(5): 893-904.

91. Battelli, M. G., A. Bolognesi & L. Polito 2014. Pathophysiology of circulating xanthine oxidoreductase: New emerging roles for a multi-tasking enzyme. *Biochim. Biophys. Acta.* 1842(9): 1502-1517.

92. Battelli, M. G. 1980. Enzymic conversion of rat liver xanthine oxidase from dehydrogenase (D form) to oxidase (O form). *FEBS Lett.* 113: 47-51.

93. Clare, D. A., B. A. Blakistone, H. E. Swaisgood & H. R. Horton. 1981. Sulfhydryl oxidase-catalyzed conversion of xanthine dehydrogenase to xanthine oxidase. *Arch. Biochem. Biophys.* 211: 44-47.

94. Li, C. & R. M. Jackson. 2002. Reactive species mechanisms of cellular hypoxia-reoxygenation injury. *Am. J. Physiol. Cell Physiol.* 282(2): C227-241.

95. Sanders, S. A., R. Eisenthal & R. Harrison. (1997). NADH oxidase activity of human xanthine oxidoreductase--generation of superoxide anion. *Eur. J. Biochem.* 245(3): 541-548.

96. Harrison, R. 2002. Structure and function of xanthine oxidoreductase: where are we now? *Free Radic. Biol. Med.* 33(6): 774-797.

97. Hanachi, N., N. Charef, A. Baghiani, *et al*. 2009. Comparison of xanthine oxidase levels in synovial fluid from patients with rheumatoid arthritis and other joint inflammations. *Saudi Med. J.* 30(11): 1422-1425.

98. Allen, R. E., J. M. Outhwaite, C. J. Morris & D. R. Blake. 1987. Xanthine oxidoreductase is present in human synovium. *Ann. Rheum. Dis.* 46(11): 843-845.

99. Stabler, T., Zura, R. D., Hsueh, M. F., & Kraus, V. B. (2015). Xanthine oxidase injurious response in acute joint injury. Clin Chim Acta, 451(Pt B): 170-174.

100. Aibibula, Z., M. Ailixiding, M. Iwata, *et al*. 2016. Xanthine oxidoreductase activation is implicated in the onset of metabolic arthritis. *Biochem. Biophys. Res. Commun.* 472(1): 26-32.

101. Stevens, C. R., M. Benboubetra, R. Harrison, *et al*. 1991. Localisation of xanthine oxidase to synovial endothelium. *Ann. Rheum. Dis.* 50(11): 760-762.

102. De La Fuente, M. & J. Miquel. 2009. An update of the oxidation-inflammation theory of aging: The involvement of the immune system in Oxi-Inflamm-Aging. *Curr. Pharm. Des.* 15(26):3003-3026.

103. Naka, K., T. Muraguchi, T. Hoshii, *et al*. 2008. Regulation of reactive oxygen species and genomic stability in hematopoietic stem cells. *Antioxid. Redox Signal*. 10(11):1883-1894.

104. Saretzki, G., T. Walter, S. Atkinson, *et al*. 2008. Downregulation of multiple stress defense mechanisms during differentiation of human embryonic stem cells. *Stem Cells*. 26(2):455-464.

105. Zhou, G., S. Meng, Y. Li, *et al*. 2016. Optimal ROS signaling is critical for nuclear reprogramming. *Cell. Reports.* 15(5):919-925.

106. Boland, M., K. Nazor & J. Loring. 2014. Epigenetic regulation of pluripotency and differentiation. *Circ. Res.* 115(2): 311-324.

107. Paul, M., B. Bisht, D. Darmawan, *et al*. 2014. Dynamic changes in intracellular ROS levels regulate airway basal stem cell homeostasis through Nrf2-dependent Notch signaling. *Cell Stem Cell*. 15(2): 199-214.

108. Volkmer, E., B. Kallukalam, J. Maertz, *et al*. 2010. Hypoxic preconditioning of human mesenchymal stem cells overcomes hypoxia-induced inhibition of osteogenic differentiation. *Tissue Eng. Part A*. 16(1): 153-164.

109. Clavijo-Cornejo, D., K. Martínez-Flores, K. Silva-Luna, *et al*. 2016. The overexpression of NALP3 inflammasome in knee osteoarthritis is associated with synovial membrane prolidase and NADPH oxidase 2. *Oxid. Med. Cell Longev.* Article ID 1472567, 7 pages.

110. Surazynski, A., W. Miltyk, J. Palka. 2008. Prolidase-dependent regulation of collagen biosynthesis. *Amino Acids*. 35(4): 731-738.

111. Altindag, O., O. Erel, N. Aksoy, *et al.* 2007. Increased oxidative stress and its relation with collagen metabolism in knee osteoarthritis. *Rheumatol. Int.* 27(4): 339-344.

112. Fernandes, J., J. Martel-Pelletier & J. Pelletier 2002. The role of cytokines in osteoarthritis pathophysiology. *Biorheology* 39(1-2): 237-246.

113. Liu, J., S. Hou, C. Tsai, *et al*. 2012. Thrombin induces heme oxygenase-1 expression in human synovial fibroblasts through protease-activated receptor signaling pathways. *Arthritis Res. Ther.* 14(2): R91-R91.

114. Hasegawa, M., T. Segawa, M. Maeda, *et al*. 2011. Thrombin-cleaved osteopontin levels in synovial fluid correlate with disease severity of knee osteoarthritis. *J. Rheumatol.* 38(1): 129-134.

115. Guillen, M., J. Megias, F. Gomar *et al*. 2008. Haem oxygenase-1 regulates catabolic and anabolic processes in osteoarthritic chondrocytes. *J. Pathol.* 214(4): 515-522.

116. Jovanovic, D., F. Mineau, K. Notoya, *et al*. 2002. Nitric oxide induced cell death in human osteoarthritic synoviocytes is mediated by tyrosine kinase activation and hydrogen peroxide and/or superoxide formation. *J. Rheumatol.* 29(10): 2165-2175.

117. Wang, J., K. Shih, Y. Wu, *et al*. 2013. Histone deacetylase inhibitors increase microRNA-146a expression and enhance negative regulation of interleukin-1beta signaling in osteoarthritis fibroblast-like synoviocytes. *Osteoarthr. Cartilage*. 21(12): 1987-1996.

118. Zhong, H., Q. Ding, W. Chen, *et al*. 2013. Vorinostat, a HDAC inhibitor, showed anti-osteoarthritic activities through inhibition of iNOS and MMP expression, p38 and ERK phosphorylation and blocking NF-kappaB nuclear translocation. *Int. Immunopharmacol*. 17(2): 329-335.

119. Ruiz-Romero, C., V. Calamia, J. Mateos, *et al*. 2009. Mitochondrial dysregulation of osteoarthritic human articular chondrocytes analyzed by proteomics a decrease in mitochondrial superoxide dismutase points to a redox imbalance. *Moll. Cell Proteomics.* 8: 172-189.

120. Roy, S. & G. Meachim. 1968. Chondrocyte ultrastructure in adult human articular cartilage. *Ann. Rheum. Dis.* 27(6): 544-558.

121. Maneiro, E., M. Martín, M. de Andres, *et al.* 2003. Mitochondrial respiratory activity is altered in osteoarthritic human articular chondrocytes. *Arthr. Rheum.* 48(3): 700-708.

122. Buczynski, M., D. Dumlao & E. Dennis. 2009. Thematic Review Series: Proteomics. An integrated omics analysis of eicosanoid biology. *J. Lipid Res.* 50(6): 1015-1038.

123. Tsolis, K., E. Bei, I. Papathanasiou, *et al*. 2015. Comparative proteomic analysis of hypertrophic chondrocytes in osteoarthritis. *Clin. Proteomics.* 12(1): 12.

124. Reed, K., G. Wilson, A. Pearsall, *et al*. 2014. The role of mitochondrial reactive oxygen species in cartilage matrix destruction. *Mol. Cell Biochem*. 397(1-2): 195-201.

125. Jeffries, M., M. Donica, W. Baker, *et al*. 2014. Genome-Wide DNA methylation study identifies significant epigenomic changes in osteoarthritic cartilage. *Arthritis Rheum*. 66(10): 2804-2815.

126. Scott, J., C. Gabrielides, R. Davidson, *et al*. 2010. Superoxide dismutase downregulation in osteoarthritis progression and end-stage disease. *Ann. Rheum. Dis*. 69(8): 1502-1510.

127. Bandiera, S., R. Matégot, M. Girard, *et al*. 2013. MitomiRs delineating the intracellular localization of microRNAs at mitochondria. *Free Radic. Biol. Med.* 64: 12-19.

128. Morita, K., T. Miyamoto, N. Fujita, *et al*. 2007. Reactive oxygen species induce chondrocyte hypertrophy in endochondral ossification. *J. Exp. Med*. 204(7): 1613-1623.

129. Lugo, L. & G. Herrero-Beaumont. 2013. "Estrogen-dependent transcriptional activity: A protection against ROS in osteoarthritis". In *Studies on Arthritis and Joint Disorders*. M. Alcaraz, O. Gualillo & O. Sánchez-Pernaute, Eds.: 369-391. New York, NY: Springer New York.

130. Blanco, F., I. Rego & C. Ruiz-Romero. 2011. The role of mitochondria in osteoarthritis. *Nat. Rev. Rheumatol.* 7(3): 161-169.

131. Soto-Hermida, A., M. Fernandez-Moreno, N. Oreiro, *et al*. 2014. MtDNA haplogroups and osteoarthritis in different geographic populations. *Mitochondrion*. 15: 18-23.

132. Shen, J., L. Feng & C. Feng. 2014. Role of mtDNA haplogroups in the prevalence of osteoarthritis in different geographic populations: A meta-analysis. *PLoS ONE*, 9(10): e108896.

133. Valdes, A. & M. Goldring. 2017. Mitochondrial DNA haplogroups and ageing mechanisms in osteoarthritis. *Ann. Rheum. Dis*. Doi: 10.1136/annrheumdis-2016-210783.

134. Lee, N., Y. Choi, J. Baik, *et al*. 2005. A crucial role for reactive oxygen species in RANKL-induced osteoclast differentiation. *Blood*. 106(3): 852-859.

135. Ha, H., H. Kwak, S. Lee *et al*. 2004. Reactive oxygen species mediate RANK signaling in osteoclasts. *Exp. Cell Res.* 301(2): 119-127.

136. Sasaki, H., H. Yamamoto, K. Tominaga, *et al*. 2009. NADPH oxidase-derived reactive oxygen species are essential for differentiation of a mouse macrophage cell line (RAW264.7) into osteoclasts. *J. Med. Invest.* 56(1,2): 33-41.

137. Mandal, C., S. Ganapathy, Y. Gorin, *et al*. 2011. Reactive oxygen species derived from Nox4 mediate BMP2 gene transcription and osteoblast differentiation. *Biochem. J.* 433(2):393-402.

138. Shi, Q., F. Vaillancourt, V. Côté, *et al*. 2006. Alterations of metabolic activity in human osteoarthritic osteoblasts by lipid peroxidation end product 4-hydroxynonenal. *Arthritis Res. Therapy*. 8(6): R159.

139. Hsu, D, P. Chu & I. Jou. 2016. Enteral sesame oil therapeutically relieves disease severity in rat experimental osteoarthritis. *Food Nutr. Res.* 60: 10.3402/fnr.v60.29807.

140. Hsu, D, P. Chu & I. Jou. 2016. Daily sesame oil supplement attenuates joint pain by inhibiting muscular oxidative stress in osteoarthritis rat model. *J. Nutr. Biochem.* 29: 36-40.

141. Cai, D., S. Yin, J. Yang, *et al*. 2015 Histone deacetylase inhibition activates Nrf2 and protects against osteoarthritis. *Arthritis Res. Ther.* 17: 269.

142. Safdar, A., J. deBeer & M. Tarnopolsky. 2010. Dysfunctional Nrf2–Keap1 redox signaling in skeletal muscle of the sedentary old. *Free Radic. Biol. Med.* 49: 1487-1493.

143. Kovac, S., P. Angelova, K. Holmström, *et al*. (2015) Nrf2 regulates ROS production by mitochondria and NADPH oxidase. *Biochim Biophys Acta* 1850: 794-801.

144. Rushmore, T., M. Morton & C. Pickett. 1991. The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J. Biol. Chem.* 266(18): 11632-11639.

145. Sporn, M. & K. Liby. 2012. NRF2 and cancer: the good, the bad and the importance of context. *Nat .Rev. Cancer.* 12(8): 564-571.

146. Cho, H., S. Reddy, A. Debiase, *et al*. 2005. Gene expression profiling of NRF2-mediated protection against oxidative injury. *Free Radic. Biol. Med.* 38(3): 325-343.

147. Malhotra, D., E. Portales-Casamar, A. Singh, *et al*. 2010. Global mapping of binding sites for Nrf2 identifies novel targets in cell survival response through ChIP-Seq profiling and network analysis. *Nucleic Acids Res.* 38(17): 5718-5734.

148. Wang, X., D. Tomso, B. Chorley, *et al*. 2007. Identification of polymorphic antioxidant response elements in the human genome. *Hum. Mol. Genet.* 16(10): 1188-1200.

149. Ahmed, S., L. Luo, A. Namani, *et al*. 2017. Nrf2 signaling pathway: Pivotal roles in inflammation. *Bba. Mol. Basis Dis.* 1863(2): 585-597.

150. Bernard, K., N. Logsdon, V. Miguel, *et al*. 2017. NADPH Oxidase 4 (Nox4) Suppresses mitochondrial biogenesis and bioenergetics in lung fibroblasts via a nuclear factor erythroid-derived 2-like 2 (Nrf2)-dependent pathway. *J. Biol. Chem.* 292(7): 3029-3038.

151. Wei, J., H. Zhu, G. Lord, *et al*. 2016. Nrf2 exerts cell-autonomous antifibrotic effects: compromised function in systemic sclerosis and therapeutic rescue with a novel heterocyclic chalcone derivative. *Transl. Res*. 16: 30422-30424.

152. Kook, S., D. Lee, E. Cho, *et al*. 2016. Activation of canonical Wnt/β-catenin signaling inhibits H2O2-induced decreases in proliferation and differentiation of human periodontal ligament fibroblasts. *Mol. Cell Biochem.* 411(1): 83-94.

153. Chang, Z., L. Huo, P. Li, *et al*. 2015 Ascorbic acid provides protection for human chondrocytes against oxidative stress. *Mod. Med. Rep.* 12: 7086-7092.

154. Davidson, R, O. Jupp, R. de Ferrars, *et al*. 2013. Sulforaphane represses matrix-degrading proteases and protects cartilage from destruction in vitro and in vivo. *Arthritis Rheum.* 65: 3130-3140.

155. Glosh, S., S. Banerjee & P. Sil. 2015. The beneficial role of curcumin on inflammation, diabetes and neurodegenerative disease: A recent update. *Food Chem. Toxicol.* 83: 111-124.

156. Kaulmann, A. & T. Bohn. 2014. Carotenoids, inflammation, and oxidative stress-implications of cellular signaling pathways and relation to chronic disease prevention. *Nutr. Res.* 34: 907-929.

157. Berenbaum, F. 2014 Does broccoli protect from osteoarthritis? *Joint Bone Spine* 81: 284-286.

158. Vos, P., J. DeGroot, A. Huisman, *et al*. 2010. Skin and urine pentosidine weakly correlate with joint damage in a cohort of patients with early signs of osteoarthritis (CHECK). *Osteoarthr. Cartilage* 18: 1329-1336.

159. Hunter, D., M. LaValley, J. Li, *et al.* 2007. Urinary pentosidine does not predict cartilage loss among subjects with symptomatic knee OA: the BOKS Study. *Osteoarthr. Cartilage* 15: 93-97.

160. Kobayashi, E., T. Suzuki, R. Funayama, *et al*. 2016. Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription. *Nature Commun.* DOI:10.1038/ncomms11624.

161. Sun, Y., L. Li, K. Corry, *et al*. 2015. Deletion of Nrf2 reduces skeletal mechanical properties and decreases load-driven bone formation. *Bone*. 74: 1-9.

162. Ibáñez, L., M. Ferrándiz, R. Brines, *et al*. 2014. Effects of Nrf2 deficiency on bone microarchitecture in an experimental model of osteoporosis. *Oxid. Med. Cell. Longev.* 2014: Article ID 726590, 9 pages.

163. Hyeon, S., H. Lee, Y. Yang, *et al*. 2013. Nrf2 deficiency induces oxidative stress and promotes RANKL-induced osteoclast differentiation. *Free Rad. Biol. Med.* 65: 789-799.

164. Kanzaki, H., F. Shinohara, M. Kajiya, *et al*. 2013. The Keap1/Nrf2 protein axis plays a role in osteoclast differentiation by regulating intracellular reactive oxygen species signaling. *J. Biol. Chem.* 288(32): 23009-23020.

165. Kanzaki, H., F. Shinohara, K. Itohiya, *et al*. 2017. RANKL induces Bach1 nuclear import and attenuates Nrf2-mediated antioxidant enzymes, thereby augmenting intracellular reactive oxygen species signaling and osteoclastogenesis in mice. *FASEB J.* 31(2): 781-792.

166. Park, C., Y. Lee, K. Kim, *et al*. 2014. Nrf2 is a novel regulator of bone acquisition. *Bone*. 63: 36-46.

167. Rana, T., M. Schultz, M. Freeman, *et al*. 2012. Loss of Nrf2 accelerates ionizing radiation-induced bone loss by upregulating RANKL. *Free Radic. Biol. Med.* 53(12): 2298-2307.

168. Lippross, S., R. Beckmann, N. Streubesand, *et al*. 2014. Nrf2 deficiency impairs fracture healing in mice. *Calcif. Tissue Int.* 95(4): 349-361.

169. Tsai, J., J. Dudakov, K. Takahashi, *et al*. 2013. Nrf2 regulates haematopoietic stem cell function. *Nat. Cell Biol.* 15(3): 309-316.

170. Tao, J., H. Wang, Y. Zhai, *et al*. 2016. Downregulation of Nrf2 promotes autophagy-dependent osteoblastic differentiation of adipose-derived mesenchymal stem cells. *Exp. Cell Res.* 349(2): 221-229.

171. Chen, X., L. Yan, Z. Guo *et al*. 2016. Adipose-derived mesenchymal stem cells promote the survival of fat grafts via crosstalk between the Nrf2 and TLR4 pathways. *Cell Death Dis.* 7(9): e2369.

172. Henrotin, Y., A. Clutterbuck, D. Allaway, *et al*. 2010. Biological actions of curcumin on articular chondrocytes. *Osteoarthr. Cartilage,* 18(2):141-149.

173. Balogun, E., M. Hoque, P. Gong, *et al*. 2003. Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. *Biochem. J.* 371(3): 887-895.

174. Su, X., Q. Huang, J. Chen, *et al*. 2016. Calycosin suppresses expression of pro-inflammatory cytokines via the activation of p62/Nrf2-linked heme oxygenase 1 in rheumatoid arthritis synovial fibroblasts. *Pharm. Res.*113(A): 695-704.

175. Khan, N., A. Hasseb, M. Ansari, *et al.* 2017. Wogonin, a plant derived small molecule, exerts potent anti-inflammatory and chondroprotective effects through the activation of ROS/ERK/Nrf2 signaling pathways in human *Osteoarthr. Chondrocytes*. DOI:10.1016/j.freeradbiomed.2017.02.041.

176. Calabrese, V., T. E. Bates, C. Mancuso, *et al*. 2008. Curcumin and the cellular stress response in free radical-related diseases. *Mol. Nutr. Food Res.* 52(9): 1062-1073.

177. Calabrese, V., C. Cornelius, A. T. Dinkova-Kostova & E. J. Calabrese. 2009. Vitagenes, cellular stress response, and acetylcarnitine: relevance to hormesis. *Biofactors*. 35(2): 146-160.

178. Calabrese, E. J., G. Dhawan, R. Kapoor, *et al*. 2015. What is hormesis and its relevance to healthy aging and longevity? *Biogerontology*. 16(6): 693-707.

179. Calabrese, V, C. Cornelius, A. T. Dinkova-Kostova, *et al*. 2012. Cellular stress responses, hormetic phytochemicals and vitagenes in aging and longevity. *Biochim Biophys Acta.* 1822(5): 753-783.

180. Haas, K., R. Watanabe, T. Matsushita, *et al*. 2010. Protective and pathogenic roles for B cells during systemic autoimmunity in NZB/WF1 mice. *J. Immunol.* 184(9): 4789-4800.

181. Tsai, P., S. Ka, J. Chang, *et al*. 2011. Epigallocatechin-3-gallate prevents lupus nephritis development in mice via enhancing the Nrf2 antioxidant pathway and inhibiting NLRP3 inflammasome activation. *Free Rad. Biol. Med*. 51(3): 744-754.

182. He, L., X. Peng, J. Zhu, *et al*. 2014. Mangiferin attenuate sepsis-induced acute kidney injury via antioxidant and anti-inflammatory effects. *Am. J. Nephrol.* 40(5): 441-450.

183. Liu, X., T. Wang, X. Liu, *et al*. 2016. Biochanin A protects lipopolysaccharide/D-galactosamine-induced acute liver injury in mice by activating the Nrf2 pathway and inhibiting NLRP3 inflammasome activation. *Int. Immunopharmacol.* 38: 324-331.

184. Yang, S., S. Ka, K. Hua, *et al*. 2013. Antroquinonol mitigates an accelerated and progressive IgA nephropathy model in mice by activating the Nrf2 pathway and inhibiting T cells and NLRP3 inflammasome. *Free Rad. Biol.Med.* 61: 285-297.

185. Lin, H., X. Gao, G. Chen, *et al*. 2015. Indole-3-carbinol as inhibitors of glucocorticoid-induced apoptosis in osteoblastic cells through blocking ROS-mediated Nrf2 pathway. *Biochem. Biophys. Res. Commun.* 460(2): 422-427.

186. Lin, H., B. Wei, G. Li, *et al*. 2014. Sulforaphane reverses glucocorticoid-induced apoptosis in osteoblastic cells through regulation of the Nrf2 pathway. *Drug. Des. Devel. Ther.* 8: 973-982.

187. Liu, W., L. Mao, F. Ji, *et al*. 2017. Icariside II activates EGFR-Akt-Nrf2 signaling and protects osteoblasts from dexamethasone. *Oncotarget.* 8(2): 2594-2603.

188. Healy, K., J. Vanhooke, J. Prahl, *et al.* 2005. Parathyroid hormone decreases renal vitamin D receptor expression in vivo. *Proc. Natl. Acad. Sci. U S A.* 102: 4724-4728.

189. Facchini, A., I. Stanic, S. Cetrullo, *et al.* 2011. Sulforaphane protects human chondrocytes against cell death induced by various stimuli. *J. Cell Physiol.* 226: 1771-1779.

190. Choi, E., K. Suh, Y. Kim, *et al*. 2016. Glabridin alleviates the toxic effects of methylglyoxal on osteoblastic MC3T3-E1 cells by increasing expression of the glyoxalase system and Nrf2/HO-1 signaling and protecting mitochondrial function. *J. Agric. Food Chem.* 64(1): 226-235.

191. Li, S., N. Chen, Y. Qiao, *et al*. 2016. SC79 rescues osteoblasts from dexamethasone though activating Akt-Nrf2 signaling. *Biochem. Biophys. Res. Commun.* 479(1): 54-60.

192. Xu, D., W. Zhao, X. Zhu, *et al*. 2016. OSU53 rescues human OB-6 osteoblastic cells from dexamethasone through activating AMPK signaling. *PLoS ONE* 11(9): e0162694.

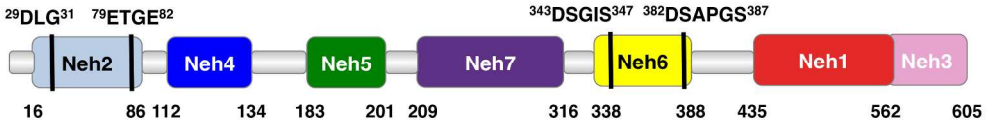

Figure 1. Domain structure of Nrf2. The relative positions of the Nrf2 ECH homology (Neh) 1–7 domains are shown. The numbering of amino acids is based on the human protein. The DLG and ETGE motifs in the Neh2 domain that bind Keap1, and the DSGIS and DSAPGS motifs in the Neh6 domain that bind β-TrCP, are indicated. The Neh4 and Neh5 domains form the transactivation domain of Nrf2 that recruits CREB-binding protein (CBP). The retinoid X receptor α (RXRα) binds to the Neh7 domain of Nrf2, leading to repression of the transcription factor. Neh1 mediates binding to DNA and to the heterodimeric partner of Nrf2, a small Maf transcription factor. Neh3 recruits the chromo-ATPase/helicase DNA-binding protein 6 (CHD6).

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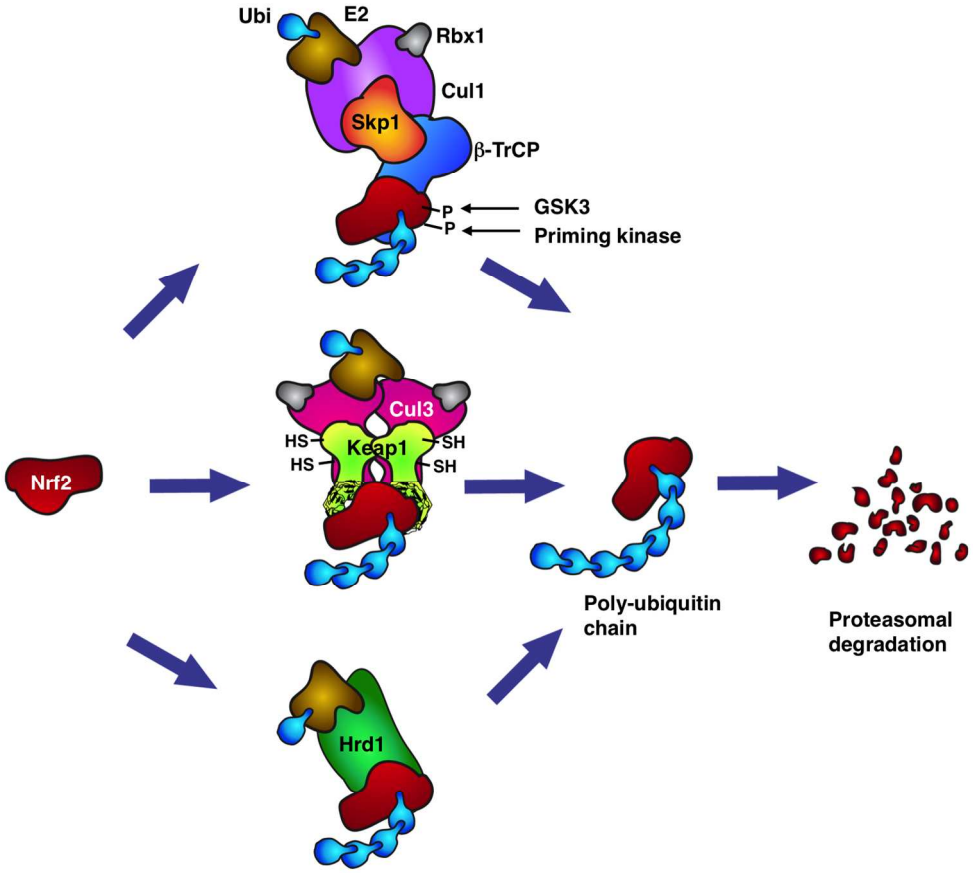

Figure 2. Regulation of Nrf2 by ubiquitination and proteasomal degradation. Three known ubiquitin ligase systems mediate the ubiquitination of Nrf2: (i) Kelch-like ECH associated protein 1 (Keap1), a substrate adaptor protein for Cullin-3 (Cul3)-Rbx1/Roc1 ubiquitin ligase (i.e. CRLKeap1), which binds through its Kelch domains to the DLG and ETGE motifs in the Neh2 domain of Nrf2. For substrate adaptor activity, the cysteine sensors of Keap1 are in reduced state; (ii) β-transducin repeat-containing protein (β-TrCP), a substrate adaptor for the Skp1-Cul1-Rbx1/Roc1 E3 ligase complex (i.e. SCFβ-TrCP). Recognition by β-TrCP requires formation of a DSGIS motif-containing phosphodegron in the Neh6 domain of Nrf2 by glycogen synthase kinase 3 (GSK3), which in turn requires phosphorylation of Nrf2 by a priming kinase; and (iii) the E3 ubiquitin ligase Hrd1/synoviolin, an endoplasmic reticulum (ER) protein. Degradation by Hrd1 has been shown to occur during ER stress.

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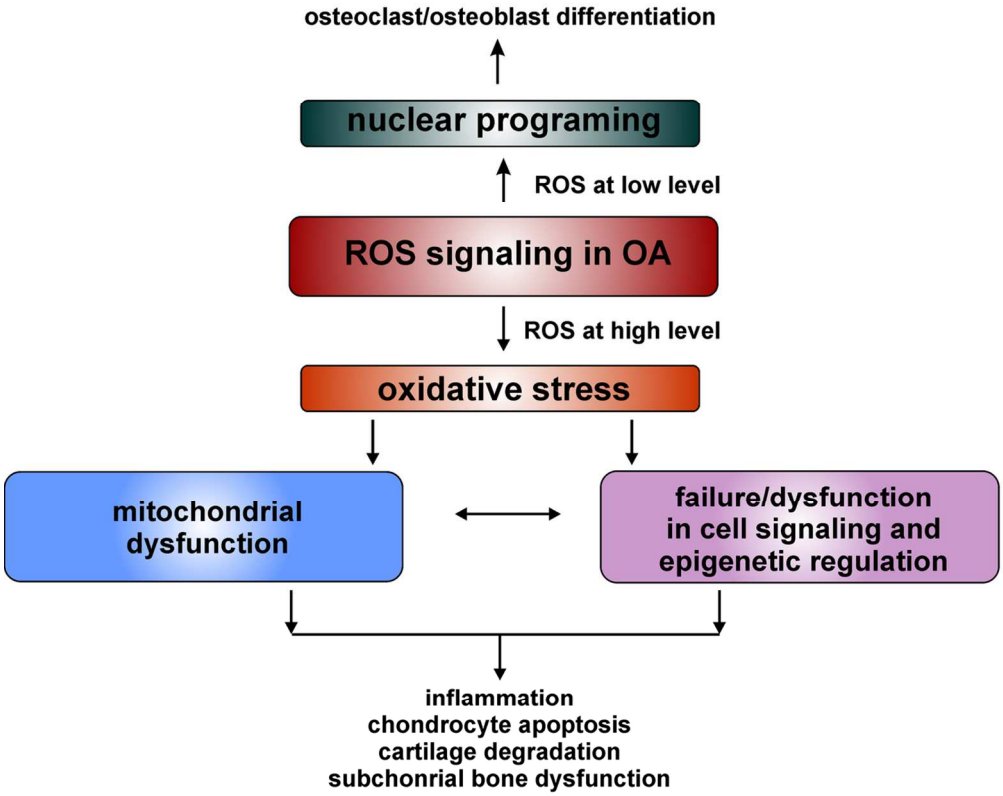

Figure 3. Role of ROS signaling in OA pathology. The low level of ROS can sustain cartilage and bone integrity as ROS regulate nuclear programing during osteoclasts and osteoblasts differentiation. At high level ROS can trigger oxidative stress with mitochondrial dysfunction and a failure in cells signaling and epigenetic regulation of gene expression. Oxidative stress causes inflammation, chondrocyte apoptosis with cartilage degradation and dysfunction in subchondral bone with imbalanced bone resorption and remodeling.

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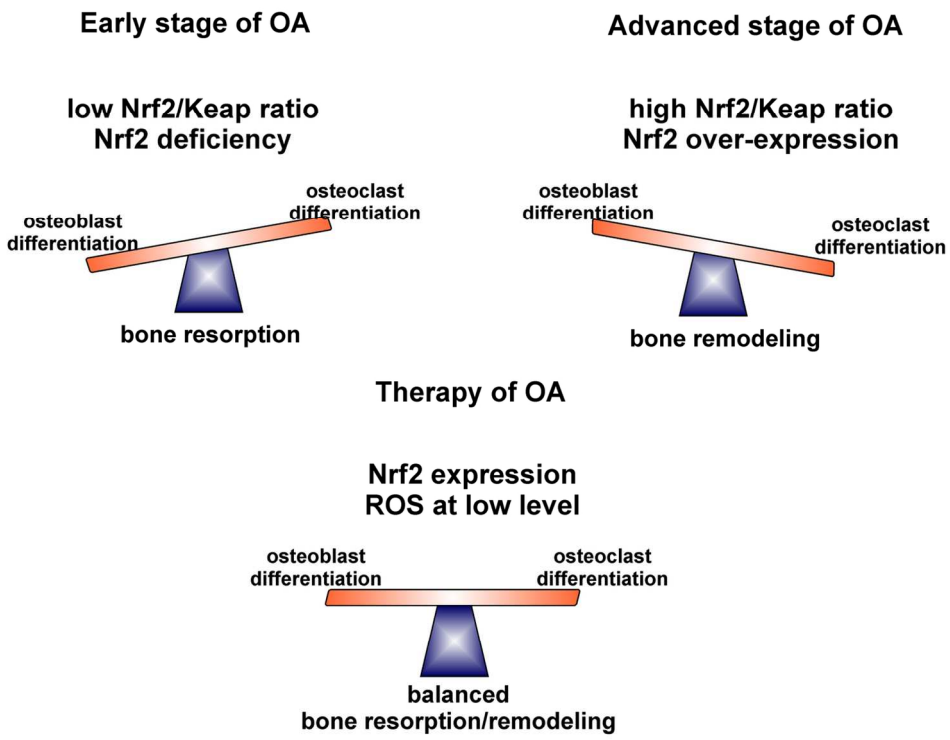

Figure 4. Role of Nrf2 in OA. Bone resorption at early stage of OA is due to a favorable osteoclastogenesis via low Nrf2/Keap ratio (as shown by Nrf2 deficiency). In advanced stage of OA, intensive bone remodeling triggered as a compensatory mechanism to bone resorption is more likely related with enhanced osteoblastogenesis via high Nrf2/Keap ratio (as shown by Nrf2 overexpression). The therapy approaches in OA may involve a normalization of bone/resorption and remodeling via interference with Nrf2 expression and ROS signaling.

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